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**EFFECT OF NANOPARTICLES ON HUMAN CELLS FROM
HEALTHY INDIVIDUALS AND PATIENTS WITH RESPIRATORY
DISEASES**

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Abstract

Ever increasing applications of nanomaterials (materials with one or more dimension less than 100 nm) has raised awareness of their potential genotoxicity. They have unique physico-chemical properties and so could have unpredictable effects. Zinc oxide (ZnO) and titanium dioxide (TiO₂) are widely used in a number of commercial products. There are published studies indicating that some forms of these compounds may be photo-clastogenic in mammalian cells. What has not been investigated before is the effect of nanoparticles from these compounds in human germ cells. Thus the present study has examined their effects in the presence and absence of UV light in human sperm and compared responses to those obtained with human lymphocytes using the Comet assay to measure DNA damage. The effect of nanoparticles (40-70nm range) was studied in human sperm and lymphocytes in the dark, after pre-irradiation with UV and simultaneous irradiation with UV. The studies do provide some evidence that there are photo-genotoxic events in sperm and lymphocytes in the absence of overt toxicity.

The cytotoxic and genotoxic potentials of ZnO and TiO₂ as well as their effect on phosphotyrosine expression, were examined in the human epithelial cervical carcinoma cells (Hela cells). This was done to try and determine the underlying molecular events resulting from their exposure to ZnO and TiO₂ nanoparticles occurring at the same time as DNA is damaged. Concentration- and time-dependent cytotoxicity, and an increase in DNA and cytogenetic damage with increasing nanoparticle concentrations were reported in this study. Mainly for zinc oxide, genotoxicity was clearly associated with an increase in tyrosine phosphorylation.

Nanotechnology has raced ahead of nanotoxicology and little is known of the effects of nanoparticles in human systems, let alone in diseased individuals. Therefore, the effects of TiO₂ nanoparticles in peripheral blood lymphocytes from patients with respiratory diseases (lung cancer, chronic obstructive pulmonary disease (COPD) and asthma) were compared with those in healthy individuals using genotoxic endpoints to determine whether there are any differences in sensitivity to nano-chemical insult between the patient and control groups. The results have shown concentration dependent genotoxic effects of TiO₂ in both respiratory patient and control groups in the Comet assay and an increasing pattern of cytogenetic damage measured in the micronucleus assay without being statistically significant except when compared with the untreated controls of healthy individuals. Furthermore, modulation of ras p21 expression was investigated. Regardless of TiO₂ treatment, only lung cancer and COPD patients expressed measurable ras p21 levels that showed modulation as the result of nanoparticle treatment.

Results have suggested that both ZnO and TiO₂ nanoparticles can be genotoxic over a range of concentrations without either photoactivation or being cytotoxic.

Key words, Nanoparticles, TiO₂, ZnO, Sperm, Lymphocyte, Comet assay, Tyrosine phosphorylation, Micronucleus assay, Respiratory diseases, lung cancer, COPD, asthma, healthy controls and ras oncoprotein.

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Abbreviations

ALS: Alkali labile site
ANOVA: Analysis of variance
APS: Ammonium persulphate
ATP: Adenosine 5' triphosphate
BNC: Binucleated cells
BSA: Bovine serum albumin
°C: Degree centigrade
CB: Carbon black
CBMN: Cytokinesis-block micronucleus
CDK: Cycline dependent kinase
CCD: Charge coupled device
CHO: Chinese hamster ovary
CNS: Central nervouns system
CO₂: Carbon dioxide
D: Dark
DMSO: Dimethyl sulphoxide
DNA: Deoxyribonucleic acid
DSB: Double strand break
DTT: Dithiothreitol
ECACC: European Collection of Cell Cultures
EDTA: Ethylene diamine tetraacetic acid
EMEM: Eagle's modified essential media
E.coli: *Echerichia coli*
FBS: Foetal bovine serum
FEV: Forced expiratory volume
G (protein): Guanosine triphosphate-coupled protein
G1 and G2: Gap phase 1 and Gap phase 2
GDP: Guanosine 5'-diphosphate
GTP: Guanosine 5'-triphosphate
HEp-2: Human epithelial cervical carcinoma (HeLa) cells

H₂O₂: Hydrogen peroxide
HO·: Hydroxyl radical
HCl: Hydrochloric acid
HPRT: Hypoxanthine phosphoribosyl transferase
kDa: Kilo-Dalton
KCl: Potassium chloride
LMP: Low melting point
M phase: Mitosis
MMC: Mitomycin C
MMP: Matrix metalloproteinase
MN: Micronuclei
ml: Millilitre
NAD: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphatase
NaOH: Sodium hydroxide
NMP: Normal melting point
NPBs: Nucleoplasmic bridges
NPs: Nanoparticles
NRU: Neutral red uptake
OD: Optical density
8-OHdG: 8 hydroxy deoxyguanosine
PAGE: Polyacrylamide gel
PBS: Phosphate buffer saline
PHA: Phytohaemagglutinin
PI: Pre- irradiated
PK: Proteinase K
PM: Particulate matter
PTK: Protein tyrosine kinase
PTP: Protein tyrosine phosphatase
P/S: Penicillin streptomycin mix
ROS: Reactive oxygen species
rpm: Revolutions per minute
RPMI: Roswell Park Memorial Institute
S phase: Synthesis phase

SD: Standard deviation
SE: Standard error
SI: Simultaneously irradiated
SPF: Sun protection factor
SDS: Sodium dodecyl sulphate
SPSS: Statistical package for social sciences
SSB: Single strand breaks
SWCNT : Single-walled carbon nanotubes
TEMED: N, N, N',N' tetramethyl ethylenediamine
TBS-T: Tris buffer solution with tween-20
TiO₂ : Titanium dioxide
μg: Micro-gram
μl: Micro-litre
μM: Micro-molar
UV: Ultra violet
v/v: Volume per volume
WHO: World Health Organization
ZnO: Zinc oxide

Chapter 1

Introduction

1. Introduction

Nanotechnology as defined by the Centre for Responsible Nanotechnology (USA) is “the engineering of functional systems at the molecular scale. It is referred to the future ability to construct items from the bottom up, using techniques and tools being developed today to make complete, high performance products” (<http://www.crnano.org/whatis.htm>).

The U.S. National Science Foundation perspective for nanotechnology is as follows:

“Imagine a medical device that travels through the human body to seek out and destroy small clusters of cancerous cells before they can spread or a box no larger than a sugar cube that contains the entire contents of the Library of Congress or materials much lighter than steel that possess ten times as much strength”. (<http://www.scribd.com/doc/18303519/Nanotechnology-for-Communications>)

The outlook for nanotechnology is to be a general-purpose technology (as it was called), because it will have significant effect on all other technologies and industries. The outcome because of nanotechnology as expected is that everything will become better. Medicines that are more effective enhanced stronger buildings, and cleaner safer water supply.

Nanotechnology is a wide field, incorporating applied physics, chemical engineering, biological engineering, etc. A nanoparticle is a small particle with a dimension generally less than 100 nm. Nanotechnology is used to manufacture polymers and in computer chip design, but the real commercial applications are in suntan lotions, cosmetics, drug

delivery and protective coatings (Hofmann-Amttenbrink et al., 2010; Nesseem, 2010; Osmond and McCall, 2010)

1.1 History of nanotechnology

It is now been realized that nanoparticles (NPs) have a very long history; they were used in the 9th century by artisans for the glittering effects or lustre on the surface of pottery. Lustre originated from films that contained copper and silver nanoparticles dispersed in a glassy matrix of ceramic. The nanoparticles used in lustre were created by adding copper and silver oxides to salts, vinegar, ochre and clay on the surface of glazed pottery then heated to 600°C. Heat would soften the glaze and make copper and silver migrate to the outer surface. The low pressures prevent the ions transforming back to the metal form and help to give the formed nanoparticles colour and optic effects. The lustre technique originated first in the Islamic world as Muslim men were forbidden from wearing gold, so they found lustre as a way to get a similar effect without using real gold.

The first scientific description of nanoparticles and their optical properties was provided by Michael Faraday in a paper in 1857 entitled “Experimental relations of gold (and other metals) to light” (Faraday 1857).

The first idea of nano-technology came from a talk given by an American physicist called Richard Feynman at the American Physical Society on December 1959. Feynman described a process by which smaller sets of atoms and molecules can be produced from manipulating larger ones (Taniguchi, 1974). Although, the term nanotechnology was defined by Professor Norio Taniguchi from Tokyo Science University in 1974 as the

process of separation, consolidation, and deformation of materials by one atom or by one molecule (Taniguchi, 1974)

In the early 1980s, nanotechnology and nanoscience started and with it came the invention of the scanning tunnelling microscope (STM). This development led to the discovery of fullerenes in 1985 and carbon nanotubes. In the year 2000, the United States National Nanotechnology Initiative was founded to coordinate Federal nanotechnology research and development (Guzman et al., 2006; Tinkle, 2010).

1.2. Properties of nanoparticles

Nanoparticles are a bridge between bulk material and atomic structures. Bulk materials have constant physical and chemical properties regardless of their size but materials reduced to the nano-size can suddenly show completely different properties (Ehrman, 1999; Hutter and Maysinger, 2010; Perrier et al., 2010; Pouliquen et al., 1991).

Agglomeration or aggregation is another definite characteristic of nanoparticles, because nanoparticles are held together by both weak and strong forces, including van der Waals and electrostatic forces, and sintered bonds. The binding force of agglomerates affects the solubility of the component of the particles under different conditions (Allouni et al., 2009; Poizot et al., 2000; Wokovich et al., 2009). Another property of nanoparticles is that as the size of the material decreases, the number of physical properties changes and subsequently becomes dominant as the nanometer size is reached. One example is the increase in surface area to volume ratio that acts as a driving force for diffusion and greater reactivity, especially at high temperatures. It also reduces the melting temperature of nanoparticles and alters most of the physical properties of the material including thermal and catalytic properties (Buffat, 1976) .

1.2.1 Photo-properties of ZnO and TiO₂

TiO₂ and ZnO nanoparticles, with sizes between 50 – 500 nm, are extensively used in cosmetics and sunscreens, mainly in the sun protection factor (SPF) fraction of sunscreen, because they act as nano-mirrors reflecting UV light and as UV filters against solar radiation. The industry took advantages of their scattering properties and small size to improve the homogeneity of distribution in cosmetics and sunscreens. Although, TiO₂ reflects and scatters UVB and UVA in sunlight, it absorbs about 70% of the total UV (Dunford et al., 1997).

Both TiO₂ and ZnO are considered as photo-catalysts. The photo-catalysis is a reaction, which uses light to activate a substance that in return modifies the rate of a chemical reaction without being involved itself and a photo-catalyst is the substance, which can modify the rate of a chemical reaction using light irradiation (Akyol and Bayramoglu, 2010; Wang et al., 2010a; Zhang et al., 2008). Chlorophyll of plants is a typical natural photo catalyst. The main difference between chlorophyll and a nano photo-catalyst is that chlorophyll converts water and carbon dioxide into oxygen and glucose in the presence of sun light, however, nanoparticles create a strong oxidation agent and electronic holes to break down the organic matter to carbon dioxide and water with the formation of an intermediate free radical, which could be harmful.

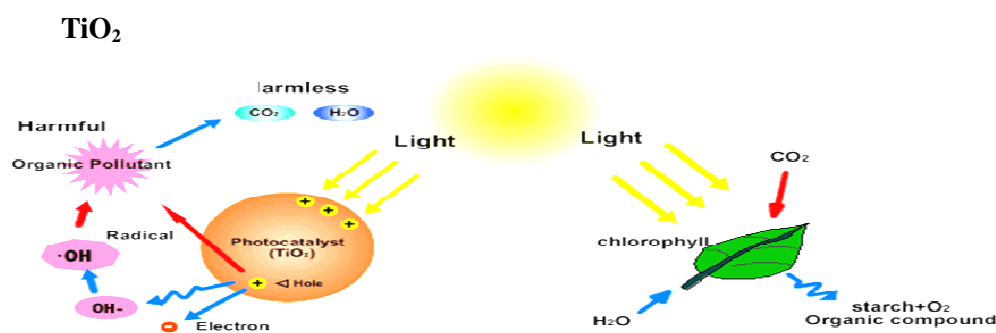


Figure 1.1 Comparison of photocatalytic property between chlorophyll and TiO₂ (from ECOSmart Australia P/L).

1.3 Applications of nanotechnology

Nanomedicine is a large industry that deals with medical applications of nanotechnology. Its sales were 6.8 US billion dollars in 2004 and with over 200 companies and 38 products worldwide (Emerich and Thanos, 2003). The uses of nanomedicines include drug delivery by using nanoscale particles to maximize bioviability of a drug at a place where it is most needed and for a prolonged time. However, the current problems for nanomedicine involve understanding the toxicity of nanoparticles (Amiji, 2010; Jin et al., 2006; Lammers et al., 2010; LaVan et al., 2003; Sheng and Huang, 2010).

In cancer, the very small size of nanoparticles (10–100 nm) and high surface area to volume ratio are very useful in cancer diagnosis for example in MRI imaging (due to size tunable light emission), where nanotechnology can produce more detailed higher contrast and lower cost images of the tumour site (Kubik et al., 2005). In addition, the small size of the NPs allows them to preferentially accumulate at the tumour sites due to lack of effective lymphatic drainage. This is a property which could be used to attach nanoparticles to future trial cancer therapies to target specific tumour cells efficiently and replace chemo and radiotherapies (Leary et al., 2006). Scientists are also investigating the possibility of manufacturing multifunctional nanoparticles which could detect, image then “cook” the tumours with radio waves that heat only the nanoparticles and their tumour cells (Brown et al., 2010; Korelitz and Sommers, 1975; Richard et al., 2008).

Other speculative claims for future medical uses of NPs include photo-dynamic therapy (Yamakoshi et al., 1999), surgery (Matteini et al., 2010), nanorobots e.g. computational genes (Cheng et al., 2010) and cell repair (Moghimi et al., 2005). However,

commercially significant uses of nanoparticles are uses in consumer products e.g. TiO_2 and ZnO nanomaterials in sunscreens, cosmetics, food products, as a white paint, outdoor furniture varnishes, surface coating and paint (Parlini, 2008). Other current and speculative household uses of nanotechnology include coating, easy to clean surfaces, in optics e.g. antireflective ultra thin sunglasses; in food for sensing and detection of biochemical changes and in textiles, nano fibres to make clothes which are stain and water repellent or wrinkle free.

Nano-filtration is another large scale product of nanotechnology through which water is produced and could be used in the developing countries which lack access to reliable water sources (Bai et al., 2010; Hillie and Hlophe, 2007; Lazarova and Spendlingwimmer, 2008).

1.3.1 Titanium dioxide (TiO_2) and Zinc Oxide (ZnO) nanoparticles

Titanium dioxide is a naturally occurring oxide of titanium, also known as titanium IV, Titania, or pigment white 6 or titanium white when used as a pigment. The chemical formula is TiO_2 . It occurs in three forms rutile, anatase and brookite. It is used, as a white pigment because of its brightness, as a reflective optical coating, in papers, foods and medicines (pills and tablets). It is widely used as a sunscreen because of its UV resistant properties and acts as a UV absorber, transforming destructive UV energy light into heat.

Nano- size ZnO has possible future uses as transparent coatings to protect against ultraviolet and infrared radiation similar in concept to the sunscreen. It could also be used in electronic applications such as photo-printing and electro-photography. Commercial uses of ZnO are: anti-corrosion coatings on steel (galvanizing),

construction material, brass, pharmaceuticals, cosmetics, and as micronutrients for humans, animals and plants.

1.3.2 Pulmonary applications of nanomedicines

Current, rapid advances in nanotechnology and nanoscience offer means of new methods of diagnosing and treating many diseases. Accordingly, in the USA, the national heart, lung, and blood institute (NHLBI) arranged a working group on nanotechnology that recommended the creation and development of research centres for applications of nanotechnology and nanoscience for heart, lung, blood, and sleep (HLBS) diseases and encouraged investigators and small businesses to develop nanotechnology-based approaches to clinical problems (Buxton et al., 2003).

Gene therapy for the treatment of lung cancer has shown promise in the laboratory and in clinical trials. However, at present its use is limited to treatment of localized lung tumours because of host-immunity in opposition to the viral gene delivery vector. Accordingly, there is a remarkable effort to develop alternative gene delivery vectors that are capable of delivering tumour suppressor genes to metastatic lung cancers and at the same time to be non-immunogenic and efficient for systemic therapy. The preclinical trials with 1,2-dioleoyl-3-trimethylammonium-propane cholesterol nanoparticles (DOTAP: Chol), which are NPs complexed to DNA (DNA-nanoparticles) have been found to be an effective such non viral alternative vector for gene delivery (Gopalan et al., 2004; Ramesh et al., 2004).

Chemotherapeutic studies have found that alginate nanoparticles used in aerosols to encapsulate the anti-tuberculous drugs isoniazid (INH), rifampicin (RIF) and

pyrazinamide (PZA) was an ideal carrier for the controlled release of anti-tuberculous drugs, because it has increased the bioavailability of all three drugs encapsulated compared to the oral free drugs. In addition, all drugs were detected 15 days post-nebulisation in the lungs, liver and spleen while the free drugs not detected after one day of administration (Ahmad et al., 2005; Alvares et al., 2008).

The use of an aerosolized nanostructure itraconazole, as prophylaxis against invasive pulmonary aspergillosis caused by *Aspergillus fumigatus*, has been investigated in mice. The nanoparticle drug itraconazole has been found to significantly limit the invasiveness of the disease and to improve mouse survival (Hoeben et al., 2006).

A novel method for systemic delivery of drugs via the lungs was reported using lecithin based nanoparticles of less than 300 nm in size in a pressurised aerosol inhaler. The NPs deposited in the alveolar space, which is an ideal deposition site for more effective results (Dickinson et al., 2001; Nyambura et al., 2009).

Another major respiratory problem that was targeted by nanomedicines is respiratory syncytial virus (RSV) infection, which is a most important cause of respiratory tract infection that has no vaccine or treatment so far existing. It is commonly associated with bronchiolitis especially in infancy with a higher risk of developing asthma later in life. Chitosan /DNA nanoparticles are synthetic polysaccharide nanoparticles commonly used in gene transfer therapy because it can release the incorporated drugs for long term. Chitosan DNA nanoparticles containing plasmid DNAs encoding all RSV antigens were used for intranasal gene transfer into mice, which have resulted in a significant induction of RSV-specific antibodies in the mice lungs with a subsequent reduction to the viral antigen load and titres following acute RSV infection. Chitosan-

DNA nanospheres were also found to reduce allergic airway inflammation similar to asthma in mice (Kumar et al., 2003).

With regard to the advances in imaging and diagnostic applications of NPs in lung imaging, these include advances in delivery of NPs imaging agents to specific tissues or cells of concern, nanoprobe for molecular imaging of the disease pathway and the enhancement in contrast agents (Buxton, 2007; Pison et al., 2006).

1.4 The lung

The main role of the respiratory system is to take oxygen from the external environment in exchange for waste gases mainly carbon dioxide, which requires the lung to work with maximum efficiency and optimise usage of the large surface area for gas diffusion. Although around 10000 litres of air are inhaled every day, the inspired air has low flow and turbulence, which facilitates particle deposition in the lungs (Coates et al., 2005; Peterson et al., 2008) .

1.4.1 Smoking and DNA damage

Various compounds in cigarette smoke can directly form radicals and produce DNA single-strand breaks; however, the procarcinogens must be activated first by P-450 cytochromes before producing single-strand breaks in the DNA, as such this could explain how mutations in some members of cytochrome P-450 are linked to lung cancer (Miller, 1978).

In vitro studies have linked tobacco smoke to DNA damage (Au et al., 1991; Bond et al., 1989; El-Zein et al., 2010; Hopkin and Evans, 1979; Hopkins and Evans, 1980; Song et al., 2010; Willey and Harris, 1990). Furthermore, an *in vivo* study has detected an increase in 8-hydroxy-2'-deoxyguanosine (which is a product of a reaction between

an oxidant and DNA) activity level in smokers' lymphocytes and lung epithelial cells (Kiyosawa et al., 1990; Leanderson and Tagesson, 1992a). Additionally, human respiratory tract epithelial cells treated with gas-phase cigarette smoke produced DNA strand breakage as well as guanine and adenine base deamination in all four DNA bases, that pattern of DNA damage was suggestive of oxygen radicals (Spencer et al., 1995). Other radicals in cigarette smoke such as hydroquinone and semiquinone have also been found to produce DNA damage which could lead to mutations and carcinogenesis (Adler et al., 1990; Yoshie and Ohshima, 1998). The damaging effect of cigarette smoking on DNA was suggested due to its ability to produce mutations in certain genes which are regarded as unique such as p53 and *K-ras* genes and are considered as fingerprints of oxidative stress to DNA and their association with higher rate and bad prognosis of lung cancer in cigarette smokers (Slebos et al., 1991).

1.4.2 Lung cancer

In the USA lung cancer is the leading cause of cancer deaths in both men and women (Greenlee et al., 2000). Lung cancer could be of primary origin from the lung cells or of secondary origin that due to metastasis from other organs such as breast, colon, prostate, cervix, etc. It is commonly presented as a persistent cough.

There are two main histological categories of lung cancer. Non-small cell lung carcinoma is accounting for about 85 % of lung cancers. It grows more slowly than the small cell lung carcinoma, and by the time of its diagnosis, 40% of the patients have metastases. The most common types of non-small cell lung carcinoma are adenocarcinoma, large cell carcinoma and squamous cell carcinoma (Trushkowsky, 1991). Small cell lung carcinoma (oat cell carcinoma), accounts for 13 % of all lung

cancers. It is very aggressive form of cancer and by the time of diagnosis the majority of patients have metastases (Goossens and De Greve, 2010).

Cigarette smoking is a major cause of lung cancers, responsible for 85% of all lung cancer and 10% of all smokers eventually develop lung cancer (Wang et al., 2010b). However, lung cancer also occurs in 10% of people who never smoked. Recent studies have found that non smokers who develop cancer have mutations in the epidermal growth factor receptor (EGFR) gene (Cappuzzo et al., 2007; Lee et al., 2010). Other risk factors include exposure to passive smoke or other carcinogens such as asbestos and radiation.

Lung cancer prognosis is always poor. People with advanced non–small cell lung carcinoma (NSCC) can survive only for 6 months. The 5-year survival rate of patients with extensive small cell lung cancer or advanced non–small cell lung cancer is less than 1% (Sawabata et al., 2010).

1.4.2.1 Genetics of lung cancer

Although there is no evidence that lung cancer has an inherited form, it has been frequently associated with some genetic mutations such as the p53 gene which is involved with Li-Fraumeni syndrome and the retinoblastoma gene (RB1). Such individuals have a higher risk of developing lung cancer or it could develop at early age. Carriers of germ-line mutations in the retinoblastoma gene are also at an increased risk (Wikenheiser-Brokamp, 2006). Additionally, a meta-analysis suggested that lung cancer incidence increases with family history of lung cancer. It more likely to occur if more than one family member has been affected and if it was diagnosed at a younger

age (Matakidou et al., 2005). However, less than 10% of all cancers are considered to be linked to genetic traits (Ludwig and Weinstein, 2005).

As some non-smokers (as well as a large minority of smokers) do develop lung cancer, genetic influences could be a possible factor of the disease. As for example mutations in some members of cytochrome P-450, GST and NAT gene families which have been found to influence detoxification of tobacco smoke constituents (Bouchardy et al., 2001) . In addition, malfunctioning of the DNA repair mechanism has also been reported in *in vivo* studies on lung cancer patient cells compared to controls (Breuer et al., 2005).

Varying mutational patterns in *K-ras* and p53 genes have also been reported in Chinese lung cancer patients. Such variation was reported between smokers and non-smokers and between different lung cancer cell types that suggest other environmental factors than smoking could be involved in the pathogenesis of lung cancer (Gao et al., 1997).

1.4.2.2 Lung cancer biomarkers

Lung cancer has become a worldwide problem as its occurrence is increasing in addition to failure of the chemo- and radio-therapeutic and surgical methods. It constantly has bad prognosis, which gives importance to the chemo-prevention therapies that are natural or synthetic small molecules targeting specific sites of mutations e.g. inhibitors of ras and epidermal growth factors, as such they can delay, prevent or modulate the carcinogenesis process (Soria et al., 2003).

Clinically available lung cancer biomarkers include carcinoembryonic antigen (CEA) (Afrikian et al., 1982) and oncofetal antigen that is elevated especially in adeno and large cell carcinomas (Boghaert et al., 2008). However, many oncogenes have been

observed and implicated in lung cancer including *K-ras*, EGFR and Myc genes (Soria et al., 2003; Sy et al., 2004; Wong et al., 2002).

1.4.3 Chronic obstructive pulmonary disease (COPD) genetics

COPD is a chronic inflammatory disease of the lower airways and lung parenchyma, which is enhanced during exacerbations. The pathological changes of COPD consist of emphysema (lung parenchyma destruction), airways inflammation, airflow limitation (reduced FEV₁) and increase in mucus-producing cells. COPD is a worldwide health problem that mostly affects people over the age of 40 years. As the disease progresses slowly most patients with symptomatic COPD are middle age or elderly and it particularly has a higher incidence in developing countries (Barnes, 2003). However, there is still a fundamental lack of knowledge about the cellular, molecular, and genetic causes of COPD, and no therapies are currently adequate to reduce COPD progression and mortality.

1.4.3.1 ROS and COPD

The oxidative stress from environmental pollutions, noxious gases and particle inhalation such as cigarette smoke has been found to play a significant role in the aging process of the lungs that leads to COPD (Buist et al., 2007; Harman, 2006; Ito and Barnes, 2009). Additionally, accumulation of ROS that formed during normal metabolism has been found to damage the DNA that can lead to aging. Such explanation is in line with the free radical theory of aging which has been supported by many subsequent studies (Galaris et al., 2008; Percy et al., 2008; Reeve et al., 2008).

1.4.3.2 Predisposing factors to COPD

Smoking is the key factor in COPD, 15% of smokers will develop COPD and smoking is responsible for 90% of COPD. The symptoms of COPD include, coughing, sputum production and breathlessness accompanied by rapid lung function deterioration. Air pollution from indoor stoves used for cooking was also reported as risk factor for COPD (Liu et al., 2008). COPD is also associated with occupational pollutants such as silica, coal miners and construction workers (Mukherjee et al., 1993).

A well-established genetic cause of COPD is alpha-1 antitrypsin (AAT) deficiency. Alpha-1 antitrypsin deficiency is a rare genetic disorder that is inherited as an autosomal recessive gene (DeMeo et al., 2009). The normal function of the lung is dependent on the elastic fibres that composed of elastin protein and surround the bronchi and the alveoli. The elastase enzyme is found in small amount in normal lungs however, its secretion increases in cigarette smokers and subsequently breaks down the elastin lung fibres and damages the bronchi and alveoli. The AAT is normally produced by the liver and is present in normal lungs to block the damaging effects of elastase.

The AAT deficiency leads to unopposed destruction of the elastic lung tissues by elastase and subsequently earlier onset of COPD which cigarette smoking may accelerate (Kellermann et al., 1991; Mangione et al., 1991).

1.4.4 Bronchial asthma

The alveolar surface of the lungs is directly in contact with the external environment. Bronchial asthma is characterized by reversible airway obstruction, increased bronchial reactivity and airway inflammation. Asthma results from complex interactions among inflammatory cells, their mediators, airway epithelium, smooth muscles and the nervous system. In genetically susceptible individuals, these interactions can lead the patient

with asthma to symptoms of breathlessness, wheezing, cough, and chest tightness (Bush, 2002). The allergic response in the airway is the result of a complex interaction of mast cells, eosinophils, T lymphocytes, macrophages, dendritic cells and neutrophils. Inhalation-challenge studies with allergens reveal an early allergic response (EAR), which occurs within minutes and peaks at 20 minutes following inhalation of the allergen. Clinically, the manifestations of the EAR in the airway include bronchial constriction, airway oedema and mucous plugging. These effects are the result of mast cell-derived mediators. Four to ten hours later, a late allergic response may occur, which is characterized by infiltration of inflammatory cells into the airway and is most likely caused by cytokine-mediated recruitment and activation of lymphocytes and eosinophils. In developed countries the prevalence of asthma has increased steadily over the last century. Current estimates suggest that about 300 million people worldwide suffer with asthma. Some reports suggest the prevalence of asthma is increasing by 50% every decade worldwide. In the United Kingdom this is more than 15% (Eder et al., 2006). Prognosis of asthma is generally good; mortality from asthma is rare and usually caused by under treatment and delay in delivering appropriate therapy. Associated phenotypes with asthma include bronchial hyper responsiveness and atopy. No asthma genes have yet been known, although, genetic studies pointed out that several genes are involved in the pathogenesis of asthma. More than 25 genes have been found to be associated with asthma or atopy (Ober and Hoffjan, 2006).

1.5 Toxicity of nanoparticles

Although free nanoparticles in the environment tend to quickly agglomerate, there still are considerable possible environmental and medical dangers because of their unpredictable behaviour especially their high surface to volume ratio which makes the particles very reactive and able to pass through cell membranes and interact with the

biological system (Bharali and Mousa, 2010; Lu et al., 2007; Maurer-Jones et al., 2010; Moore, 2006; von der Kammer et al., 2010). Nanomaterials can enter human tissues through different ports, for example, via the lungs after inhalation (Oberdorster, 2001), through the digestive system (Jung et al., 2000) and possibly through the skin (Kreilgaard, 2002; Lademann et al., 1999). Nanoparticles have also been found to reach the olfactory bulb and the cerebellum crossing the blood–brain barrier (Borm and Kreyling, 2004; Oberdorster et al., 2004). NPs could also cross the blood-testis barrier (Braydich-Stolle et al., 2005; Jani et al., 1990).

1.5.1 Nanoparticles and generation of oxidants

The genotoxicity of NPs in general could be primary and inflammation-independent (Schins, 2002; van Maanen et al., 1999) or secondary to inflammation (Greim et al., 2001). NPs could induce ROS and RNS which lead to DNA damage (Marnett, 2000) as well as to oxidative attack of the DNA, and therefore leading to structural changes in the DNA, for example to deletions, insertions or base pair mutations which are common in mutated oncogenes such as *ras p21* and tumour suppressor genes (Wiseman and Halliwell, 1996).

As such, persistent formation of oxidants that occurs as the result of inflammations that are induced by various particles is considered as a major factor in nanoparticle induced genotoxicity. The major oxidants that are inducing as a result of NPs are hydroxyl radicals ($\bullet\text{OH}$) because they are produced mainly as a result of the Fenton reaction which involves hydrogen peroxide and transition metals. The ($\bullet\text{OH}$) are the most potent ROS that could react with the four DNA bases and generate new products (Lloyd et al., 1998; Pryor, 1988).

1.5.1.1 The lung and generation of oxidants

Previous studies have shown the ability of the lung epithelial cells to generate ROS after exposure to quartz particles, fly ash or PM through processes involving mitochondrial respiration and activation of NADPH (Deshpande et al., 2002; Driscoll et al., 2001; Li et al., 2003; Shukla et al., 2000; Voelkel et al., 2003). Nanoparticles may also activate intracellular oxidant formation within the lung such as ROS and RNS which are endogenously generated by the lung epithelial cells or vascular endothelial cells (Arroyo et al., 1990; Guo et al., 1995; Kinnula et al., 1991). However, the most important ROS/RNS generating system in the lung is the influx of inflammatory phagocytes during particle exposure (Porter et al., 2002; Schapira et al., 1995). In addition to phagocytes, neutrophils and macrophages could also be activated involving NADPH in a process in which oxidase activation has demonstrated in studies with asbestos, silica, coal fly ash and ambient particulate matter (PM) (Becker et al., 1996; Gusev et al., 1993; Hedenborg and Klockars, 1989; Leanderson and Tagesson, 1992b; Prahalad et al., 1999).

1.5.2 Mechanisms of toxicity of nanoparticles

Reactive oxygen species (ROS) are believed to play a major role in the primary genotoxicity of nanoparticles as they generate free radicals in cells and cause oxidative stress. These contribute to their ability to cause cell injury, inflammation, and genotoxicity. Excessive and persistent formation of ROS from inflammatory cells is considered the hallmark of genotoxicity of nanoparticles. For example, studies on coal fly ash have shown a role of particulate size and ion release in radical generation and oxidative DNA damage (Kleijnans et al., 1989).

Particle size, shape, crystallinity (e.g., silica), solubility and interaction with the cell division process (e.g., asbestos) are other factors besides, the presence of mutagens carried with the particle (e.g., diesel exhaust particles, DEP), are additional factors related to the primary genotoxicity of nanoparticles.

Photo-mutagenicity, which is the alteration of a non-mutagenic chemical into another which is likely to cause mutation after or during exposure to UV light, is another mechanism of the toxicity of nanoparticles. The best known photo-mutagens are the furocoumarins especially 8-methoxypsoralen or 8-MOP (Ashwood-Smith et al., 1982; Borges et al., 1998; Kim et al., 2000; Miolo et al., 1999; Mizuno, 1981; Song and Tapley, 1979).

The nanoparticle's small size is the most studied contributor to NPs toxicity. For instance in a study that supported the size mechanism of NPs toxicity, treatment of a human bronchial epithelial cell line with anatase TiO_2 (10 and 20 nm) resulted in DNA damage, lipid peroxidation and micronuclei formation in the absence of photo-activation through oxidative stress induction, however; treatment of the same cells with anatase-sized (200 and > 200 nm) particles did not induce oxidative stress (Gurr et al., 2005). In a study in bacteria that was comparing toxicity of nano size metal oxides, titanium dioxide, zinc, aluminium and silicon to that of their counterpart larger size particles, the nano-scaled ZnO have been found to cause bacterial mortality of up to 100% to all three tested bacterial strains, followed by aluminium and silicon but not titanium dioxide. Such toxicity was species specific and higher in the nano-scaled than the bulky counterparts; the study suggested that toxicity has occurred as response to the dissolved particles oxide that could explain the lesser toxicity of TiO_2 because it tends to aggregate (Jiang et al., 2009). Additionally, earlier studies have shown that lung

epithelial cells exposed to different nano-sized particles induced cell proliferation which was related to small particles and the bigger particles failed to induce inflammation, it was suggested that the special surface properties developed as a consequence of the extremely small size of the particles was responsible for the cell proliferation (Baulig et al., 2003; Brunsgaard, 1998; Unfried et al., 2008).

Particle solubility has also contributed to NPs toxicity. For example *in vitro* cytotoxicity tests, evaluating the effect of seven industrial nanoparticles on human mesothelioma and in a rodent fibroblast cell line, it was found that solubility strongly influenced NPs cytotoxicity (Schrand et al., 2010). In a different *in vitro* study involving metal oxide toxicity, it was found that cytotoxicity could be contributed to by the dissolved metal ions (NPs dissolution) before or after the NPs entered the cell for example Zn^{2+} (Brunner et al., 2006).

1.5.3 Pulmonary toxicity of nanoparticles

Occupational and environmental exposure to many particles has been related to an increasing risk of lung diseases and lung cancer such as crystalline silica and asbestos fibres. The mechanism of nanoparticle genotoxicity to the lung could be due to reactive oxygen species generated primarily by NPs through different mechanisms such as mitochondrial activation by the NPs or secondary through induction of an inflammatory response, as well as NPs carrying adsorbed carcinogenic components into the lung. Pulmonary effects of inhaled NPs are attributed to the ease of their translocation from the lung into the blood stream due to their small size and their capacity to produce inflammatory responses in the lung.

There are many NPs to which the lung could be exposed. A substantial number of nanoparticles are already in consumer products such as sunscreens, cosmetics, and many

more to follow. There is great potential for the respiratory system to be open to countless unique nanoparticles. *In vivo* studies of dispersed single-walled carbon nanotubes (SWCNT) have been found to rapidly be incorporated into the alveolar interstitium and subsequently increased collagen deposition (Mercer et al., 2008). The kinetics of distribution of intravenous administered TiO₂ in male Wistar rats treated for 1, 14, and 28 days distribution, then assessed for toxicity and distribution has found that, TiO₂ has maximum availability in the liver, followed by spleen, lung, and kidney with no toxic effect and not detected in blood cells or brain (Fabian et al., 2008).

In vivo studies in rats exposed to aggregated ultra fine TiO₂ and carbon black, have found that both compounds induced lung tumors in rats at considerably lower gravimetric lung burdens than larger sized TiO₂ (Bermudez et al., 2002; Oberdorster, 1996). In another *in vivo* study, rats developed more lung inflammation with a nano size TiO₂ compared to that produced by larger particles (Donaldson et al., 2002; Oberdorster et al., 2000). Yet another *in vivo* study, has found that rats exposed to ultra-fine P25 TiO₂ for a period of time, produced lung tumours in 16-30% of the studied rats which indicates that chronic inhalation of the studied nanoparticles can be carcinogenic (Heinrich et al., 1995).

Significant species differences too have been reported in the pulmonary responses to inhaled TiO₂ particles, for example rats developed a more severe and persistent pulmonary inflammatory response than mice or hamsters and were also unique in the development of progressive fibro-proliferative lesions and alveolar epithelial metaplasia in response to exposure to a high concentration of TiO₂ particles (Bermudez et al., 2002). As regards to the photo-genotoxic effects of TiO₂ to the lungs, TiO₂ have been found to induce clastogenicity in Chinese hamster lung cells (Nakagawa et al., 1997) in

the Comet assay, but positive responses were generally only at toxic doses. However, TiO₂ was found not to exhibit photochemical genotoxicity in the same system in another study (Theogaraj et al., 2007).

Environmental studies have suggested that low exposure to ambient particulate matter (PM), contributes to lung cancer, even after correction for other risk factors such as cigarette smoking, diet, occupational exposure and other individual risk factors (Abbey et al., 1999; Pope et al., 2002). PM is a mixture of particulate matter from different sources therefore, PM is subject to continuous variation in composition and activity but it is not clear which of the PM components could be responsible for its carcinogenicity (Salvi and Holgate, 1999). In addition, nanomaterials are increasingly emitted in the environment due to the large scale of nanomaterials production which has been shown to be responsible for many acute and chronic disorders including neoplasia.

Different mechanisms have been suggested to contribute to NPs induced lung diseases including particle accumulation followed by inflammatory cell influx and subsequently cell proliferation. Also NPs directly or indirectly through inflammatory cell production can cause genetic damage or the production and release of oxidants which could lead to proliferative or mutational effects to their target cells. Accumulation of mutated cells could eventually cause malignant diseases particularly if protective mechanisms such as DNA repair and selective apoptosis fail. The 8 hydroxydeoxyguanosine (8-OHdG), which is an oxidized nucleoside of DNA that occurs upon DNA repair, was found to be increased in the lung tissues of rats exposed to diesel exhaust inhalation (Ichinose et al., 1997; Seiler et al., 2001). In addition, *in vivo* studies in rats exposed to carbon black or to crystalline silica have shown enhanced *HPRT* mutations in alveolar epithelial cells (Driscoll et al., 1997).

Oxidative stress is now considered as a mechanism underlying inflammatory, proliferative and genotoxic responses as well as regulating cellular signalling due to ability of the particles to generate reactive oxygen species (ROS) and reactive nitrogen species (Fubini and Hubbard, 2003; Schins, 2002).

1.5.4 Toxicity to other systems

1.5.4.1 Dermatological toxicity

Skin plays a critical role in the testing for toxicity of nanoparticles, as sunscreens constitute most of the commercial use of nanoparticles as part of the sun protection factor (SPF), which contain up to 25% of either ZnO or TiO₂. On testing TiO₂ (anatase and rutile) and ZnO extracted from different sunscreens in cultured human fibroblasts, the results showed that illuminated TiO₂ induced DNA strand breaks, and there was some evidence that hydroxyl radicals may have played a part in the observed DNA effects. This was because DMSO and mannitol suppressed the DNA damage, and the conclusion of the study suggested possible health hazards with TiO₂ if it can enter viable cells after penetrating the stratum corneum (Dunford et al., 1997). Studies carried out *in vitro* and *in vivo* on human cells, found that TiO₂ caused formation of hydroxyl (\bullet OH) radicals with subsequent DNA damage (strand breaks) through the generation of free radicals by photo-catalytic reactions .

However, in a second but contradictory study, human volunteers and skin biopsies were tested for dermal penetration of coated TiO₂ micro particles, the result showed that the deeper the skin the more the skin free from NPs. The TiO₂ concentration as well was decreased with increasing depth of the stratum corneum (Lademann et al., 1999). In a

third contradictory study in which different skin samples were used, electron microscopy results showed that in healthy skin the nanoparticles penetrated into the deepest corneocyte layer of the skin, but never reached the vital layer (Kertész *et al.* 2004). In addition, on testing dermal uptake of nano TiO₂ and determining its localization in the skin using transmission electron microscopy (TEM), TiO₂ was found only in trace amounts in the upper part of the follicle as clear agglomerates without any evidence of uptake into follicular epithelium. The study concluded that follicles are not a relevant route of penetration for TiO₂ (Pflucker *et al.*, 1999).

1.5.4.2 Haematological and cardiovascular toxicity

Nanoparticles of gold and polystyrene have been shown to cause haemolysis and blood clotting. Combustions e.g. diesel soot and diesel particles injected into hamster lungs caused clear cardiovascular effects and thrombosis (Nemmar *et al.*, 2001). Additionally, inhaled nanoparticles, were also found to interfere with the heart pace maker directly, causing heart rate alteration and arrhythmias in hypertensive rats (Campen *et al.*, 2003). Furthermore, carbon black nanoparticles directly injected into the blood of healthy mice, induced platelet accumulation in hepatic micro vessels with prothombotic changes (Khandoga *et al.*, 2004).

1.5.4.3 Central nervous system (CNS) toxicity

After inhalation, NPs can bypass the blood brain barriers and reach the brain through the olfactory epithelium (Lockman *et al.*, 2004), or could be carried out wrapped in low density lipoproteins (Kim *et al.*, 2007). A healthy blood brain barrier (BBB) protects the brain from the effects of nanoparticles but some diseases, as for example hypertension, distort BBB defences and increase their permeability of the barriers. Also the surface charges of nanoparticles alter the BBB rendering it more permeable and less

effective and subsequently nanoparticles can get easy access to the brain (Lockman et al., 2004). Additionally, studies in mice exposed to nanoparticles showed activation of pro-inflammatory cytokines in the brain (Fabian et al., 2008; Shin et al., 2010).

1.5.4.4 Germ cell toxicity

DNA damage to somatic cells can give rise to cancer but damage to germ cells can not only give rise to cancer, but can also cause congenital defects in future generations. Studies in Chinese hamster ovary (CHO) cells treated with ZnO under various illumination conditions, found that ZnO has photo-clastogenic properties which were four-fold greater in the presence of UV light, but less than the photo-genotoxic effect of 8-MOP (Dufour et al., 2006) .

In addition, a separate study has found that gold nanoparticles had spermatotoxic effects after gold nanoparticles mixed with human spermatozoa lead to gold penetration into the sperm heads and tails and as a result, 25% of sperm lost their motility. The effect of different types of nanoparticles was also investigated in mouse spermatogonial germ line stem cells and demonstrated a concentration-dependent toxicity for silver, molybdenum and aluminium nanoparticles tested, whereas the corresponding soluble salts had no significant effects (Braydich-Stolle et al., 2005).

1.5.4.5 Bactericidal effect of nanoparticles

The bactericidal action of photo-activated TiO₂ particles have been demonstrated on contaminated dental implants and metal pins used for skeletal traction (Tsuang et al., 2008). In addition, TiO₂, SiO₂ and ZnO suspended in water have been found to be cytotoxic towards all tested bacteria (*B. subtilis* and *E. coli*) and the eukaryotic *Daphnia*

magna. In addition, the toxicity of NPs increased with the particle concentrations and decreased from ZnO to TiO₂ to SiO₂ (Adams et al., 2006a; Adams et al., 2006b).

On the other hand, several studies have reported that fullerenes nC60 have bactericidal effects on gram negative and gram-positive bacteria and other studies reported almost complete elimination of bacteria from water by using nC60 (Fortner et al., 2007; Lyon and Alvarez, 2008). Not all nanoparticles are bactericidal, but modified fullerenes are used as antimicrobial agents.

In addition, Z-MITE powders, which are inorganic zinc oxide nanoparticles, are used commercially as antibacterial and antifungal and they confirm the cytotoxic effect of ZnO.

1.5.4.6 Toxicity to environmental aquatic species

There is a great concern regarding the environmental impact of nanoparticles, in the form of adverse effects on wildlife. Studies showed harmful and damaging effects to wild life creatures. A study examined water-soluble fullerene (nC60) and it has been shown to induce lipid peroxidation and oxidative stress in the brain of the juvenile largemouth bass (LMB) fish (Oberdörster, 2004). Water-stirred-nC60 was found to elevate lipid peroxidation in the brain and gill of aquatic fish significantly causing 100% mortality (Zhu et al., 2006). The aquatic toxicity screening studies in a 72h acute test involving the green algae *Pseudokirchneriella subcapitata* have concluded that ultra-fine TiO₂ has a low hazard potential (Warheit et al., 2007a). However, (Zhu et al., 2008) have studied developmental toxicity of metal oxide nanoparticles, and the results demonstrated that ZnO is very toxic to zebra fish embryos and larvae. Photo-activated TiO₂ extracted from different sunscreens, have been reported to catalyse DNA damage both *in vitro* and in human cells (Dunford et al., 1997).

1.5.4.7 Cellular effects of nanoparticles

Previous studies have shown that Chinese Hamster V79 cells exposed to micronised ZnO, developed structural chromosomal aberrations. The researcher concluded their findings as a positive clastogenic activity of ZnO under the study conditions (Theogaraj et al., 2007). In another study, 130 µg/ml of TiO₂ in the absence of UV light, were found to be highly toxic to WIL2-NS cells (Wang et al., 2007b). The potential use of TiO₂ in the photo killing of malignant cells in the presence of photo-irradiation (photodynamic therapy), was investigated in HeLa cells treated with illuminated ultra fine TiO₂ powder, the researchers reported that HeLa cells were completely killed after TiO₂ exposure and the resulting cytotoxicity was due to free radical formation (Ito and Barnes, 2009).

1.6 DNA repair mechanisms

DNA damage results in chromosomal breakage, alteration in the gene transcription of the cell to which that specific DNA encodes and induction of mutations in the cell genome. Therefore, the DNA repair mechanisms are essential to maintain the genome stability and integrity, thus the DNA repair is a continuously active process through which the cell identifies and corrects the damage to the DNA in its genome. In human cells, as many as 1000 - 1 million lesions per cell occurs every day as a result of normal metabolic activities or environmental factors such as UV light, radiation and other genotoxins (Browner et al., 2004). The cell responds to genotoxic insults by activating different pathways such as cell-cycle arrest, apoptosis or DNA repair. Many factors govern the rate of DNA repair, these include the cell age and type. Subsequently the cell that has a great amount of accumulated DNA damage, or the cell that cannot repair the DNA damage effectively can either enter a state of senescence (which is a state of irreversible dormancy, apoptosis or undergoes uncontrolled cell division with

neoplastic formation especially if it occurred in critical genes as for example the tumor suppressor genes. The DNA damage repair genes protecting the DNA and as such influence the life span.

1.6.1 Single strand DNA repair

For single strand breaks (SSBs), the repair mechanism involve one strand to be used as a template for the correction of the damaged strand through different excision repair mechanisms mostly by replacing the damaged nucleotide with an undamaged complementary nucleotide from the undamaged DNA strand (Watson and Losick, 2004). The base excision repair (BER) is an example of the excision repair mechanism, which repairs damage to a single DNA base that damaged through a different biochemical processes. The damaged part is removed by a DNA glycosylase, the endonuclease then recognise the missing base which is then resynthesized by DNA polymerase, and sealed up by DNA ligase (Watson and Losick, 2004). Mismatch repair (MMR), is another repair mechanism that corrects undamaged but mispaired nucleotides due to errors of DNA replication and recombination (Watson and Losick, 2004).

1.6.2 Double strand DNA repair

DNA repair is also critical for cell survival after mammalian cells exposure to genotoxic agents that cause DNA damage especially DSBs. The double-strand breaks and DNA crosslinkages that occur as the result of failed normal repair system and or apoptosis are particularly dangerous because they can cause genome rearrangements (Acharya, 1972; Bjorksten et al., 1971).

The DNA repair mechanisms for double-strand breaks (DSBs), includes two pathways, in the first pathway the DNA broken ends aligned and joined together by sequence homology, in the second pathway the broken ends rejoined without sequence homology:

1) Non-homologous end joining (NHEJ): In NHEJ, DNA Ligase IV together with cofactor XRCC4 act together to directly joins the two broken DNA ends (Wilson et al., 1997) and for accurate repair, NHEJ rely on a short homologous sequence (microhomologies) at the ends of a single-strand tail to be joined. If these microhomologies are compatible, they usually give accurate repair although it can accidentally produce mutations for example deletion due to loss of the damaged nucleosides during repair (Burma et al., 2006)

2) Homologous recombination requires the presence of identical or semi-identical sequences because that sequence will be used as a template for the damaged chromosome repair through a sister chromatid which would be available in G2 (Watanabe et al., 2009).

Defects in DNA repair (faulty DNA repair) can leads to accumulation of mutations and genomic rearrangements that promote carcinogenesis or various hereditary genetic disorders such as hereditary cancers e.g. breast and hereditary nonpolyposis colorectal cancer (HNPCC), xeroderma pigmentosum and all accelerated aging diseases in which their sufferers appears old and suffer from geriatric diseases at young age. Inherited DNA repair genes mutations are associated with high cancer risk for example BRCA1 and BRCA2 genes which are tumour suppress genes that helps in DNA repair, BRCA1 and BRCA2 mutations leads to hereditary breast cancer (Boulton, 2006).

1.7 The cell cycle controls and cancer

Autosomal cell cycle normally pass through four phases: S phase which is the DNA synthesis and replication phase and M phase (mitosis) or cell separation phase. The S and M phases are separated by two gap phases G_1 and G_2 and the non dividing cell rest at G_0 phase the whole cell cycle machinery is dependent on cycline kinases (CdKs) activation or inhibition (Hamel and Hanley-Hyde, 1997).

Tumours can be either benign or malignant "cancer". Malignant tumours grow aggressively and invade other tissues of the body (metastasis), as the result tumour cells enter into the bloodstream or lymphatic system and then invade other sites in the body. All cancers result from an abnormality in the balance system of the cell growth. Normally, balance between cell growth and cell death is maintained so that the cells division to produce new cells occurs only when new cells needed. Disruption of the control system that checks and balance cell growth results in an uncontrolled cell division and eventually tumour formation. Mutations which enhance cell proliferation and that inhibit apoptotic signals leads to cancer.

In the last decades, a number of mutated genes have been identified in human cancer cells, and categorized into: proto-oncogenes, tumour suppressors and DNA repair genes. Mutational changes in these genes by activation of proto-oncogenes or inhibition of tumour suppress genes can lead to uncontrolled cell growth, proliferation and immortality. In addition, mutation of DNA repair gene can lead to inactivation and accumulation of mutations.

1.7.1 Ras proteins

Human *ras* genes code for small proteins those contain 189 amino acids, with a molecular weight of 21,000 Daltons (p21). The ras proteins are members of GTPases family (G proteins), that include proteins involved in signal transduction in which ras

act as a molecular switch because it alternate between an inactive form guanosine 5'-diphosphate (GDP) and an active guanosine 5'-triphosphate (GTP) form (Boguski and McCormick, 1993). *Ras* is synthesized as a biologically inactive (Pro-ras) and localized to the inner surface of the plasma membranes (Hancock et al., 1989; Hancock et al., 1990; Pearson, 1979). *Ras* genes which have yet been identified are structurally and functionally similar three ras oncoproteins named *H-ras* gene, which is homologous to the oncogene of the Harvey murine sarcoma virus, the *K-ras* gene (homologous to the oncogene of the Kirsten murine sarcoma virus and the *N-ras* gene (Mulcahy et al., 1985).

1.7.1.1 Ras role in the cell cycle regulation

Previous studies have shown that stimulation of ras p 21 expression in quiescent mouse fibroblasts lead to cell cycle entry, and an antibody to ras prevents S phase entry (Stacey and Kung, 1984). Ras proteins were shown to influence cells proliferation, differentiation, transformation, and apoptosis by signalling mitogenic and growth signals into the cell cytoplasm and nucleolus (Khosravi-Far and Der, 1994). During the cell cycle ras have been found to become activated in the mid G₁ phase (Taylor and Shalloway, 1996), it also involve in multiple steps of transition between G₁ and S phases (Dobrowolski et al., 1994). For normal cell growth to occur, it needs balance between growth stimulation and growth inhibition pathways. Malfunction of numerous pathways is needed for malignant transformation.

1.7.1.2 Ras p21 and cancer

The ras p21 protein encodes by the *ras* gene which was the first oncogene shown to cause human cancer (Barbacid, 1987) through single base changes in the ras gene which turn ras proteins to transforming protein (p21). Ras p21 contains substitution of single

amino acids at position 12, 13, and or 61 (Croce, 2008) in the wild-type, which are critical positions in the polypeptide chain (Bos, 1989; Pincus, 2004). The *ras* oncogenes in different human tumors contain such point mutation, which leads to their transforming activity that can induce neoplasm in experimental animals (Chie et al., 1999; Stanley, 1995).

Mutated *ras* genes have been found in around 30% of human malignancies, with the highest incidence of *ras* gene mutations reported in *K-ras* genes, which was detected in 80-90% of pancreatic malignancies (Ballas et al., 1988; Smit et al., 1988) and in 30-60% of colorectal carcinomas (Husgafvel-Pursiainen et al., 1992; Rodenhuis et al., 1997; Rodenhuis and Slebos, 1992; Sagawa et al., 1998; Suzuki et al., 1990). Many studies targeted *ras* in the last two decades to have better understanding of the mechanism by which mutated *ras* gene confer into cells neoplasia which could help in the production of more effective and less toxic cancer therapeutic. In quiescent normal cells GTPase regulatory activating proteins (GAPs) help to keep most p21 *ras* in an inactive GDP, over expression of non-mutated *ras* could lead to GAPs regulatory proteins saturation with subsequent deregulation of *ras* activity and oncogenic transformation (Ahmadian et al., 1996).

1.7.1.3 Ras and other signalling pathways

The non-mutated *ras* genes (wild type) play a key role in the regulation of many physiological processes, such as a role as intermediates in other signal transduction pathways that act as shuttles between the cytosol and the nucleus (Adler et al., 1995; Adler et al., 1996). These signalling pathways include receptor tyrosine kinase and other protein kinase which control many cellular processes, such as cellular growth and

differentiation and apoptosis (Barbacid, 1987; Boguski and McCormick, 1993; Bos, 1989; Lowy and Willumsen, 1993; Ruta et al., 1986)

Activated, ras p21 stimulates wide range of essential proteins that regulates different nuclear and cytoplasmic processes, such as growth factors (e.g. epidermal growth factor) and hormones e.g., insulin (McCormick, 1994). For ras to induce mitosis, it must activate other proteins that end in the nucleus, typically, tyrosine kinase is the cell-surface receptor for these growth factors which in return promote autophosphorylation (Bos, 1989; Khosravi-Far and Der, 1994; McCormick, 1993; Pazin and Williams, 1992). However, the most important of such proteins is Raf which becomes activated after it binds to ras-p21 in the cell membrane, Raf in return activates many phosphorylation reactions which includes activation Mitogen-activated protein kinase (MAPK).

1.7. 2 Phosphotyrosine and the cell cycle

Tyrosine is a non- essential amino acid, which, is normally used by the cell for protein synthesis. It is also called (Tyr or Y) or 4 hydroxyphenylalanine, tyrosine is found naturally in casein and acts as a precursor of important hormones and enzymes e.g. adrenaline and thyroxin. Most importantly it has a key function in the signal transduction process as it acts as a receiver of phosphate groups by protein kinases (receptor tyrosine kinases). Phosphorylation of the hydroxyl group changes the activity of the tagged protein (phosphorylated) and is referred to as phosphotyrosine.

Various tyrosine kinases (TK) are turned on during cell division while non-proliferating cells have very low levels of tyrosyl phosphorylated proteins. As such phosphotyrosines are a very important subset of proteins that mediates a variety of cellular processes, including cell differentiation, growth, adhesion, motility, death, and

metabolism. Disturbances or dysregulations of tyrosine phosphorylation is implicated in the development of many human diseases, such as diabetes and cancer (Krause and Van Etten, 2005). Phosphotyrosine play a key role in a variety of cellular functions and have been the targets for investigation of specific signalling pathways as well as for the development of therapeutic drugs.

1.8 Methods for the study of genotoxic effects

1.8.1 The Comet assay

The Comet assay or single cell gel electrophoresis assay is a sensitive method compared that can detect double strand breaks (DSB), single strand breaks (SSB), alkali labile sites (ALS) and cross-links. It is a reliable, rapid method for DNA damage detection that can reliably assesses and determine *in vitro* and *in vivo* environmental genotoxins (Collins and Horvathova, 2001; Singh et al., 1988; Tice et al., 2000).

The alkaline Comet assay with pH ≥ 13 for DNA unwinding and electrophoresis, has advantages over the neutral Comet assay (pH 8-9) as it allows the detection of double DSB, SSB as well as ALS, while the latter can mainly detect DSB (Singh et al., 1988) and only some SSB from the relaxation of super coiled loops. The alkaline comet tail is formed of SSB and alkali labile sites but mainly single strand breaks (Collins, 2004).

Many chemicals, medicines as well as foods were investigated for their genotoxicity in lymphocytes and sperm either on their own (Anderson et al., 1997), or combined with antioxidants (Anderson et al., 2003). Some chemicals for example, ethylene glycol mono ethyl ether produced positive responses in sperm but not in lymphocytes, which was thought to be due to lack of repair in sperm compared to lymphocytes (Anderson et al., 1997).

The US Food and Drug Administration (US FDA), and the UK Committee on Mutagenicity of Chemicals in Food, Consumer Products and Environment have approved the use of the Comet assay for *in vivo* bio-monitoring of genotoxins (Burlinson et al., 2007). For example in mice herbicides like bentazon were investigated (Garagna et al., 2005) and in rats cyclophosphamide (Anderson et al., 1996). The Comet assay has also been used in sperm to test people exposed to occupational hazards from pesticides (Xia et al., 2008).

1.8.1.1 Comet assay on lymphocytes

Lymphocyte cells are routinely used as surrogate to test for different chemicals and other genotoxic materials. All lymphocytes should be tested for viability after treatment to exclude cytotoxic effects of the chemicals, and viability of $\geq 75\%$ in the trypan blue dye exclusion test should be produced for the tested compound to avoid false positive results on the Comet assay (Henderson et al., 1998). Viability varies and 50% viable cells in trypan blue are considered as viable by the World health organization (World Health Organization, 2001). However, 75% was preferred as a cut-off point to avoid false positive (Henderson et al., 1998). Untreated lymphocytes can show between 0-10% DNA in the tail (Collins, 2004) and sperm can show up to 20 % DNA in the tail (McKelvey-Martin et al., 1997).

1.8. 1.2 Comet assay on sperm

To investigate repro-toxins with the Comet assay, sperm have more advantage over ova as they are easier to obtain by simple non invasive methods, also sperm production is continuous and renewable throughout most of human life (Baumgartner et al., 2009).

Compared to lymphocytes, sperm chromatin contains nine fold more single strand segments which subsequently could become ALS. Also, sperm nuclei contain more

than double the SSB compared to lymphocytes (Muriel et al., 2004). Sperm chromatin is 6 times more compacted than metaphase chromosomes (Ward and Coffey, 1991). Sperm from fertile men are more resistant to toxicants than infertile samples (McKelvey-Martin et al., 1997), which has been indicated by completed questionnaires from donors, including their reproductive history, according to the WHO criteria (World Health, 2001). Sperm DNA also contains highly condensed nuclear chromatin stabilized by intra- and intermolecular disulphide bonds (Hughes et al., 1996). Breaking the disulphide bond during lysis is an important step for a successful alkaline Comet assay ($\text{pH} \geq 13$) (Singh et al., 1989). In the alkaline Comet assay ($\text{pH} \geq 13$) human and mouse sperm can yield up to 10^6 - 10^7 SSB due to the presence of numerous alkali labile sites which is a functional characteristic of sperm, and does not occur under neutral conditions nor in lymphocytes (Singh et al., 1989). Depending on the conditions used, sperm freezing can affect sperm chromatin structure and morphology due to ice crystal formation or severe osmotic changes (Hammadeh et al., 1999). It can also increase membrane rigidity and decrease viability to $\leq 45\%$. However other studies found no differences between fresh and frozen sperm samples (Steele et al., 2000).

1.8.2 Western blotting method

Western blotting or immunoblotting allows the determination, with a specific primary antibody, the relative amounts of the protein present in different samples. Tyrosine phosphorylation of proteins is difficult to detect unless external stimuli are present; even then, many proteins are phosphorylated only in response to one stimulus (Ignatowski, 2001).

1.8.3 The cytokinesis-block micronucleus assay (CBMN)

The cytokinesis-block micronucleus assay (CBMN) in human lymphocytes is a commonly used assay for measuring DNA damage that occurs as a result of different genotoxic and cytotoxic insults (Fenech, 2007), in determining safety of pharmaceuticals and chemicals and in preventive medicine (Bonassi et al., 2007; Fenech et al., 2005; Kirsch-Volders et al., 2003; Lee et al., 2003). The many advantages of CBMN compared with other cytogenetics methods lead to it being preferred over the other cytogenetic methods. These advantages include that it is a quick, inexpensive method and it is easier to score micronuclei compared with chromosome aberrations (Fenech, 2002b). It is also easy to identify the cells that have successfully completed one nuclear division, because micronuclei, nucleoplasmic bridges and nuclear buds are dependent on and only expressed after completion of nuclear division (Umegaki and Fenech, 2000).

The CBMN can be carried out in human blood or lymphocytes after the cell cultures are stimulated for cell division (PHA). It could also be carried out on cell lines. Measurements should be carried out in cultured and/or mammalian cells because scoring is restricted to BN cells that denote dividing cells (Carter, 1967; Fenech, 2000).

In the CBMN, the DNA damage is measured in a divided binucleated (BN) cell and the measurements include scoring the number of micronuclei (MN) which are biomarkers of chromosome breaks or whole chromosome loss, nucleoplasmic bridges (NPBs) which are biomarkers of DNA misrepair that measure chromosome rearrangement and nuclear buds are biomarkers of amplified DNA repair complexes, in addition to apoptotic and necrotic cells (Shimizu et al., 2000). The mechanism of MN, NPBs and nuclear buds occurs at the anaphase of nuclear division, chromosome fragments or

whole chromosomes that lag behind give rise to micronuclei (Fenech, 2000; Heddle, 1973; Schmid, 1975).

In this assay, the dividing cell cytokinesis is blocked with cytochalasin B (Cyto B), which is an inhibitor of microfilament ring assembly that is essential for the completion of the cell division therefore the cell division is blocked in the binucleated stage (Fenech, 2000; Fenech and Morley, 1986). The micronuclei originate during cell division as a result of chromosome fragments or whole chromosomes that failed to accompany the mitotic spindle, accordingly it lags behind and therefore, expressed as micronuclei. Additionally, prevention of anaphase bridges breakage as a result of cytokinesis inhibition by cyto B makes it accessible to score nucleoplasmic bridges (NPBs) that originate through a variety of mechanisms. Nucleoplasmic bridges could originate from centromeres of dicentric chromosomes or chromatid that pulled to the opposite end of the cell leading to asymmetrical chromosome rearrangement or DNA strand breaks misrepair (Thomas et al., 2003) or telomere end fusions that occurs as a consequence of telomere shortening accompanied aging process (Blasco, 2005; Stewenius et al., 2005). Moreover, detection of nuclear buds, which is a marker of possible gene amplification and an indicator of genotoxic insult similar to MN and NPBs, is another advantage of CBMN assay (Fenech, 2002a; Serrano-Garcia and Montero-Montoya, 2001).

1.9 Aims and objectives:

- In spite of the fact that ZnO and TiO₂ are widely used in many consumer products, none of the investigations has focused on the effects of these nanoparticles on human germ cells. Therefore the first plan was to investigate

the effect of ZnO and TiO₂ (anatase form) in human sperm and lymphocytes in the presence and absence of UVA irradiation using the Comet assay.

- Different mechanisms of ZnO and TiO₂ toxicity were previously considered in many studies however, tyrosine phosphorylation which is a major signalling pathway that mediates cell growth, differentiation, host defence, and metabolic regulations, was never investigated before. Hence the second strategy was to investigate the tyrosine phosphorylation events in the presence of different ZnO and TiO₂ concentrations in HEp-2 cells (HeLa). Additionally, to investigate their cytotoxic (using MTT and NRU assays) and genotoxic (using Comet and CBMN assays on the same cell line.
- Given the scale of nanotechnology production, therefore, it is inevitable that the waste of nanotechnology products will accumulate and contaminate the environment. The respiratory pathway is the main route of entry of environmental pollutant particles to the body through inhalation. Accordingly, it is necessary to understand how engineered TiO₂ NPs interact with human cells following exposure of the more vulnerable respiratory disease patients compared to the healthy individuals. Therefore, the objective was to compare the effects of TiO₂ nanoparticles in peripheral blood lymphocytes from patients with respiratory diseases and healthy individuals using genotoxic endpoints (Comet and CBMN assays) and to determine whether the respiratory disease patients had a specific mechanism, which could contribute to TiO₂ potential toxicity.

Chapter 2

Materials and Methods

2.1 Materials

The following chemicals were used in the work, obtained from suppliers indicated.

Table 2.1: Chemicals and their suppliers

Chemicals, Reagents And Media	Supplier
Acetic acid	BDH, Poole, UK
Acrylamide / Bis solution 30%	Bio-Rad, Hertfordshire, UK
Agarose normal melting point	Invitrogen, Paisely, UK
Amersham Hyperfilm ECL	GE Healthcare, Buckinghamshire , UK
Ammonium persulphate(APS)	Sigma, Poole, Dorset, UK
Anti-biotin, HRP-linked antibody	Cell signalling, Hitchin, Hertfordshire, UK
Anti mouse IgG, HRP-linked antibody	Cell signalling, Hitchin, Hertfordshire, UK
Biotinated protein ladder	Cell signalling, Hitchin, Hertfordshire, UK
Bovine serum albumin	Invitrogen Ltd, Paisley, UK
Bromophenol blue	Sigma, Poole, Dorset,UK
Coumaric Acid (p-)	Sigma, Poole, Dorset, UK
Cytochalasine B	Sigma, Poole, Dorset, UK
DC Protein Assay Kit	Bio-Rad, Hertfordshire, UK
Dimethyl sulfoxide (DMSO)	Sigma, Poole, Dorset, UK
Dithiothreitol (DTT)	Sigma, Poole, Dorset, UK
EMEM- EBSS culture medium	Invitrogen Ltd, Paisley, UK
Ethanol	BDH, Poole, Dorset, UK
Ethidium bromide	Sigma, Poole, Dorset, UK
EDTA	BDH, Poole, Dorset, UK
Fetal calf Serum	Invitrogen Ltd, Paisley, UK
Formaldehyde	Merck, Nottingham, UK
GADPH	Abcam, Cambridge, UK
Giemsa staining solution	BDH, Poole, Dorset, UK
Glutamine	Gibco, Paisley, UK
Glycerol	Sigma , Poole, Dorset, UK
HEp-2 (HeLa derivative)	ECACC, Porton Down, Salisbury, UK

Histamount	Fisher scientific, Loughbrough, UK
Hybond-P (PVDF membrane)	GE Healthcare, Buckinghamshire, UK
Hydrogen peroxide	Sigma, Poole, Dorset, UK
Laemli sample buffer 2X concentrate	Sigma, Poole, Dorset, UK
Low melting point agarose	Sigma, Poole, Dorset, UK
Luminol	Sigma, Poole, Dorset, UK
Lymphoprep	Axis Shield, Oslo, Norway
Mitomycin C	Sigma, Poole, Dorset, UK
2-Mercaptoethanol	Sigma Aldrich, York, UK
Methanol	Sigma, Poole, Dorset, UK
Non Essential Amino Acids	Gibco, Paisley, UK
Pan ras p21 primary antibody	Abcam, Cambridge, UK
Phosphatase inhibitor cocktail 2	Sigma, Poole, Dorset, UK
Phospho-tyrosine mouse mAb	Cell signalling, Hitchin, Hertfordshire, UK
Protease inhibitor cocktail	Sigma, Poole, Dorset, UK
Phytohaemagglutinin (PHA)	Invitrogen Ltd, Paisley, UK
Ponceau S, practical grade	Sigma, Poole, Dorset, UK
RPMI medium 1640	Invitrogen, Paisley, UK
Sodium dodecylsulphate	Sigma, Poole, Dorset, UK
Sodium Chloride	BDH, Poole, Dorset, UK
Sodium Hydroxide	BDH, Pool, Dorset, UK
Supported nitrocellulose membrane 0.45 µm	Bio-Rad, Hertfordshire, UK
SW 480 cell lysates	Abcam, Cambridge, UK
Titanium dioxide iv (anatase)	Sigma, Poole, Dorset, UK
N,N,N',N'-Tetramethylethylenediamine (TMED)	Sigma, Poole, Dorset, UK
Triton X-100	Sigma, Poole, Dorset, UK
Trizma base	Sigma, Poole, Dorset, UK
Trypan blue	Sigma, Poole, Dorset, UK
Trypsin	Sigma, Poole, Dorset, UK
Tween 20	Sigma, Poole, Dorset, UK
Zinc oxide	Sigma, Poole, Dorset, UK

Table 2.2 Equipment and other materials

Equipments and other Services	Company / Distributor
Centrifuge Mistral 3000	MSE, Albertville, USA
Centrifuge (biofuge 28 RS)	Heraeus, Sepatech, Germany
CCD camera	Hitachi KPMI/EK Monochrome, Japan
Coplin jar	VWR, Lutterworth, UK
Cover glass	VWR, Lutterworth, UK
Cryovials	Sarstedt, Numbercht, Germany
Culture flasks (25&75 Cm ³)	Corning Incorporated Co star [®] , NY, USA
Dry incubator (37° C) LKB BIOCHROM	Leec LTD, Nottingham, UK
Electrophoresis power supply	Consort (E861), Belgium
Electrophoresis tank (HU20)	Scie-Plas, Renfrewshire, UK

Falcon tubes	BD, Swindon, UK
Freezer -20° C	Sanyo, Ultra low, Japan
Freezer -80° C	Sanyo, Ultra low, Japan
Fluorescent microscope	Leica, Weztler, Germany
Fume cupboard	Milton, UK
Fume hood ray air	Maiche Aire, Bolton, UK
Ice maker (Scotsman AF 100)	Namur, Belgium
Incubator 37° C with 5% CO ₂	Hera cell 150, Heraeus, Germany
Light microscope	Nikon, Japan
Liquid nitrogen tank	Biostar, UK
Komet 4 soft ware	Kinetic Imaging, Nottingham, UK
Magnetic stirrer SMI	Stuart Scientific, Essex, UK
Microcentrifuge MSE	GMI, Alberville, USA
Microcentrifuge MSE	Sanyo, Bucks, UK
Microplate reader	Dynex technology, Sussex, UK
Microscope (ortholux)	Leitz, Sturtgart, Germany
Mini protein 11 gel electrophoresis	Bio- Rad, Hertfordshire, UK
MSE 150 WATT ultrasonic disintegrator MK2	MSE technical, Bucks, UK.
Pipettes	Gilson, Middleton, WI, USA
pH meter	Dunmow, UK
Power pack supply	Pharmacia LKB, Uppsala, UK
Quanta 400 scanning electron microscope	FEI, Netherlands
Quartz Cuvete	Vector, Peterbrough, England
Quartz Cuvete reader	Pharmacia Biotech Ltd.,Cambridge, UK
Spectrophotometer Biochrom ultrospec 11	LKG
Super frost slides	VWR, Lutterworth, UK
Ultra spec 2000 spectrophotometer Biochrom ultrospec 11	Pharmacia Biotech Ltd.,Cambridge, UK
UV light Lamp	UVitec, Cambridge, UK
Water bath	Grant instruments, Cambridge, UK
Zeta sizer-nano	Malvern instruments, UK

2.2 Methods:

2.2.1 Methods for effect of zinc oxide and titanium dioxide nanoparticles in the Comet assay with UVA photoactivation of human sperm and lymphocytes study

2.2.1.1 Preparation of ZnO and TiO₂ nanoparticle stock suspensions

Zinc oxide (ZnO) CAS 1314-13-2, Sigma Aldrich) and titanium (IV) oxide nano-powder 99.7%, anatase (CAS 1317-70-0 Sigma Aldrich), were suspended in DMSO and used to make serial dilutions. After suspension of Zinc oxide (ZnO) and titanium dioxide (TiO₂ anatase) nano-powders in DMSO, They were sonicated using a MSE

ultrasonic disintegrator (MK2 -150 Watt) for 5 min (1 min on and 30 seconds off). Before filtration the optical density (OD) at optimum wavelengths of 370 nm for ZnO and 340 nm for TiO₂ was measured using a spectrophotometer. The suspensions were filtered through a 0.2 µm syringe filter and the OD of the filtrate measured. Using the OD before and after filtration, the concentrations of the filtrate were determined. To prepare the stock suspensions that were used in the experiments, the filtrates were processed through serial dilutions. Four concentrations were used for each of the compounds tested. For ZnO, the concentrations used were 11.5, 46.2, 69.4 and 92.3 µg/ml and for TiO₂ the concentrations used were 3.73, 14.92, 29.85 and 59.7 µg/ml. Concentrations were different due to the differential solubility of these two compounds in DMSO. The particle size was ascertained using photon correlation spectrometry (Zeta analyser, Malvern instruments) and scanning electron microscopy (SEM). For SEM analysis a drop of nano-suspension of the ZnO and TiO₂ was placed and air dried on separate mesh Formvar carbon coated (adhesive carbon stubs) and then used for SEM. The particle size was observed to be between the 40 nm and 70 nm range.

2.2.1.2 Blood sample collection and lymphocyte isolation

The collection of lymphocytes had been approved by the University of Bradford Subcommittee for ethics in research involving human Subjects Ref no. 0405-8. Blood samples were collected in 10 ml heparinized tubes from healthy non-smoker individuals after signing a consent form. Following blood sample collection, the following method was used to isolate the lymphocytes

1. The blood sample was diluted 1:1 in 0.9% NaCl and mixed gently
2. LymphoprepTM (3 ml) was placed in 15 ml Falcon tubes.

3. Each 3 ml LymphoprepTM were then overlaid gently and carefully (so as not to mix the phases) with 6 ml of the diluted blood.
4. Falcon tubes were centrifuged at 1900 rpm (800g) for 20 minutes.
5. In the meantime, 10 ml of 0.9% NaCl (w/v) were placed in 50 ml universal tubes
6. Four layers of different blood consistencies were formed and the cloudy layer (lymphocytes) was collected from the interface by a Pasteur pipette and transferred to 50 ml Universal tubes.
7. The Universal tubes were then centrifuged at 1500 rpm (400g) for 15 minutes at 20°C.
8. The supernatant was removed and the pellet was resuspended in 900 µl warm foetal bovine serum (FBS) and placed in a cryovial supplemented with 100 µl DMSO. The cryovials were placed at -20° C for 2 hours and transferred to -80° C overnight and lastly placed in liquid nitrogen.

2.2.1.3 Single cell gel electrophoresis (SCGE)/ Comet assay

The alkaline Comet assay $\text{pH} \geq 13$ was used in all experiments according to the methods by Tice and Singh (Singh et al., 1988; Tice et al., 2000). In this technique DNA damage is measured cell by cell using a micro gel electrophoresis technique. The image/ shape of the damaged migrating DNA under the microscope resemble a comet.

The alkaline Comet assay $\text{pH} \geq 13$ for DNA unwinding and electrophoresis was first introduced by Singh in 1988. It is a sensitive, reliable and rapid and can detect double strand breaks (DSB), single strand breaks (SSB), alkali labile sites (ALS) and cross-linked DNA damage (Wang et al., 2000). Additionally it can reliably assesses and

determine in vitro and in vivo lifestyle and environmental genotoxins (Baumgartner et al., 2009).

In the alkaline Comet assay, the comet tail is formed of SSB and ALS and mainly SSB (Collins, 2004) and has advantages over the neutral Comet assay (pH 8-9) as it allows detection of double DSB, SSB as well as ALS, while the latter can mainly detect DSB and only some SSB from the relaxation of super coiled loops (Singh et al., 1988).

Table 2.3 Chemicals and reagents for the Comet assay

Buffers/Reagents	Chemical constituents of reagents
Low melting point agarose (LMP)	0.5% (w/v) low melting point agarose in PBS 1% (w/v) low melting point agarose in PBS 2% (w/v) low melting point agarose in PBS
Normal melting point agarose	1% (w/v) normal melting point agarose in ddH ₂ O ₂
Lysing buffer	2.5 M NaCl, 8g NaOH, 100 mM EDTA, 10 mM Trizma base dissolved in ddH ₂ O ₂ then the pH is adjusted to 10. Triton X-100 and DMSO added later.
Sterile PBS	In one litre ddH ₂ O ₂ dissolve the following 8g NaCl, 0.2g, KCl, 1.44g Na ₂ HPO ₄ , 0.24g KH ₂ PO ₄ , and adjust pH to 7.4 with 0.1M HCl, then autoclaved.
Neutralizing buffer	0.4 M Trizma adjust pH to 7.5 with concentrated HCl
Staining solution	20 µg/ml ethidium bromide (EtBr)
Electrophoresis buffer	10 M NaOH , 1 mM EDTA (pH 10 with NaOH or HCl

2.2.1.3. 1 Alkaline Comet assay on lymphocytes

2.2.1.3.1.1 Treatments and viabilities

All membrane integrity/ viabilities were measured by trypan dye blue exclusion to avoid false positive responses due to toxicity. The cut-off point used was 75% viable cells that exclude trypan blue dye due to their intact membranes which indicate viability (Henderson et al., 1998). For lymphocytes: Viabilities were measured in freshly collected as well as thawed samples. However, the viabilities for sperm were measured only in fresh samples, as it is difficult to obtain viabilities after freezing. Different concentrations of all chemicals used in this study were tested for their cytotoxicity after treatment and before applying such concentrations; only cells showed viability of $\geq 75\%$ were used. The steps followed for trypan exclusion test were as follows:

After treatment and before performing the Comet assay or adding LMPA, all treated cells were tested for viability at the concentrations chosen to exclude cytotoxic effects. The viability was determined by the trypan dye exclusion test (10 μ l of 0.05% trypan blue was added to 10 μ l of cell suspension) and the percentage of cells which excluded the dye (indicating an undamaged cell membrane) was estimated using a haemocytometer under a microscope. Only concentrations with viability of $\geq 75\%$ in the trypan blue dye exclusion test were considered for the test compound to avoid false positive results (Henderson et al., 1998). Different concentrations of ZnO, TiO₂ H₂O₂ were prepared, and then tested on lymphocytes and sperm for viability. Only concentrations that gave viability $> 75\%$ were used in all experiments whenever H₂O₂ used (Henderson et al., 1998) .

2.2.1.3.1.2 DNA damage and repair of H₂O₂ in lymphocytes

Different H₂O₂ concentrations were prepared and tested on lymphocytes viability by trypan blue exclusion test only H₂O₂ concentrations that gave lymphocytes viability of $\geq 75\%$ was selected for the experiment. The experiment was carried out as follows: For

lymphocytes resuscitation, PBS was prepared in a universal tube; lymphocytes were thawed quickly and added to PBS. The cells were washed in PBS by centrifugation at 1600 rpm for 5 minutes to remove DMSO and FCS, because DMSO is an antioxidant and FCS proteins are also protective to the cell and act as a protein shield around the cell. The supernatant was discarded and the pellet re-suspended in PBS.

Phosphate buffered saline (PBS) 890 µl was used as a solvent, 10 µl treatment solution (1%) and 100 µl lymphocyte solution were mixed in Eppendorf® tubes and incubated at 37°C for 30 minutes. After incubation the Eppendorf® tubes were centrifuged at 4000 rpm for five minutes, then 900 µl of supernatant was discarded, the cell pellet was re-suspended and mixed with an equal volume of 1% low melting point agarose (Invitrogen: 15517-022) and layered on super frost slides. The layer was allowed to gel on an ice block. A third layer of 0.5% low melting point agarose was added on top and also allowed to gel on an ice block, the Comet assay was then carried out.

For the repair kinetics study, 900 µl of the supernatant were removed and replaced with RPMI 1640. The cells were gently resuspended and incubated at 37°C for different times up to 20 hours. There after slides were prepared and Comet assay was carried out.

2.2.1.3.1.3 UVA irradiation of lymphocyte cells

Three sets of 6 well cell culture plates (CLS 3516: Corning® Incorporated Co Star® NY, USA) were used for each experiment. The UVA radiation dose was provided using a lamp which consisted of two Waldmann F15/T8-PUVA tubes (Villingen-Scwenningen, Germany) in a casing (Wavelength range : 320-410 nm with peak emissions at 351 nm). In each set, a negative control with PBS in place of the chemicals and a positive control (80µM H₂O₂) were included for comparison. Each experiment was carried out with 3 independent repeats. In each experiment the following chemical

concentrations were used: for ZnO, (0, 11.5, 46.2, 69.4 and 92.3 $\mu\text{g/ml}$) and 80 μM H_2O_2 (as a positive control) and for TiO_2 the concentrations used were (0, 3.73, 14.92, 29.85 and 59.7 $\mu\text{g/ml}$) and 80 μM H_2O_2 (as a positive control).

Phosphate buffered saline (PBS) 890 μl was used as a solvent, 10 μl treatment solution (1%) and 100 μl lymphocytes solution were mixed in Eppendorf[®] tubes and incubated at 37°C for 30 minutes in H_2O_2 assays and for different times for nanoparticles depending on the assays. After incubation the Eppendorf[®] tubes were centrifuged at 4000 rpm for five minutes and after discarding 900 μl of supernatant, the cell pellet was re-suspended and mixed with an equal volume of 1% low melting point agarose (Invitrogen: 15517-022) and layered on super frost slides. The layer was allowed to gel on an ice block. A third layer of 0.5% low melting point agarose was added on top and also allowed to gel on an ice block.

2.2.1.3.1.3.1 Non-irradiation of lymphocytes (D)

A 6-well cell culture plate with PBS (phosphate buffered saline) and cells was floated in a water bath in the dark (without UVA irradiation) maintained at 37°C for 30 min after which the chemical was added and further incubated in the dark maintained at 37°C another 30 min.

2.2.1.3.1.3.2 Pre-irradiation (PI) of lymphocytes

The second 6-well cell culture plate with PBS and the cells was floated in a water bath maintained at 37°C for lymphocytes and exposed to UV for 30 min (1.68 milliwatts/cm²). Subsequently the chemical was added and incubated for another 30 min in the absence of UV at 37°C.

2.2.1.3.1.3.3 Simultaneous irradiation (SI) of lymphocytes

The third 6-well cell culture plate with PBS and the cells was floated in a water bath maintained at 37°C in the dark for 30 min. After this the chemical was added and exposed to UV for 30 min (1.68 milliwatts /cm²) at 37°C. In each set, a negative control with PBS in place of the chemicals and a positive control (80 mM H₂O₂) were included for comparison.

2.2.1.3.1.4 Slide preparation

The slides were prepared according to the protocol of (Tice et al., 2000). Super frosted slides flamed in 70% ethanol were dipped in 1% normal agarose (Invitrogen: 15510-027) and left to air dry to be used in the Comet assay. Following incubation of lymphocytes with the different chemicals, the samples were centrifuged. An amount of 900 µl was then removed from the supernatant. The remaining 100 µl of the cell samples (approx 10⁴) was mixed with 100 µl of 1% LMPA. The second layer of the LMPA was spread over 2 duplicate slides and immediately covered with a cover glass (24 x 50 mm) and left to set on ice tray for 5 minutes. A third layer of 0.5% LMPA (100 µl) was applied on top of the second layer after carefully removing the cover slips and left to dry on an ice for 5 minutes.

2.2.1.3.1.5 Lysis of lymphocyte cells

The aim of the lysing step was to liberate the DNA. After removal of the cover slips the slides were immersed laterally in cold lysing solution. The lysing solution contained high salts to accelerate cell swelling and rupture the membrane. It consisted of 2.5M NaCl (BDH), 100 mM EDTA (Sigma: E 3154), 10 mM Trizma base (Sigma: T- 6066), 10% DMSO and 1% Triton X-100. The lysing solution was prepared during the incubation period, for each 50 ml, 44.5 ml lysis solution, 0.5 ml Triton X-100 and 5 ml DMSO were used. DMSO was used to prevent radical induced DNA damage

associated with iron released from erythrocytes during lysing. The lysing step started after the third layer of agarose solidified, the cover slip was removed and the slides were immersed laterally and incubated over night in a cold final lysing solution at 4°C which was chilled to maintain the stability of the agarose layers.

2.2.1.3.1.6 DNA unwinding and electrophoresis of lymphocytes

The slides removed from the lysing solution were rinsed carefully in electrophoresis buffer $\text{pH} \geq 13$ to remove detergent and salts. The slides were kept in an electrophoresis tank filled up to the level of ~ 0.25 cm above the slides with electrophoresis buffer [containing 60 ml of 300 mM NaOH (BDH: 301675N) and 1mM Na₂ EDTA (pH ~ 13.5)] and 1930 ml of ddH₂O₂ for 30 minutes at 4°C to produce single stranded DNA and to express alkali labile damage. Unwinding and electrophoresis was conducted at 4°C for 30 minutes each for lymphocytes or 20 minutes each for sperm using a constant voltage of 25 volts /cm and ~ 300 mA current, which was adjusted by raising or lowering the buffer level and using a compact power supply. Unwinding and electrophoresis were conducted inside a fridge to avoid light.

2.2.1.3.1.7 Neutralization

The slides were removed from the electrophoresis buffer and neutralized with three washes of cold Tris buffer (pH 7.5) which contained Trizma base with the final concentration of 0.4 M. The aim of the neutralization step was to stop the alkaline buffer effect.

2.2.1.3.1.8 Slide staining and coding

In this assay ethidium bromide (EtBr) which is a DNA specific dye was used to visualize the slides at a concentration of (20 µg/ml), 60 µl were added to each slide. All slides were coded by an independent person to prevent bias in scoring.

2.2.1.3.2 Comet assay on sperm

The collection of sperm had been approved by the University of Bradford Subcommittee for ethics in research involving human Subjects Ref no. 0405-8. The semen samples were obtained from healthy volunteers through masturbation after abstinence for 4 days. All donors completed a questionnaire including their reproductive history and conformed to the WHO criteria for collection of sperm (World Health, 2001). The sperm samples were deposited at the laboratory within 2 hours of collection. A 100 µl aliquot was used for conventional semen analysis and 50 µl of semen was aliquoted into Eppendorf® tubes and flash frozen in liquid nitrogen and stored in liquid nitrogen for future use. Comet assay on sperm was carried out according to the protocol of (Anderson et al., 2003).

2.2.1.3.2.1 Effect of H₂O₂ on sperm

Different H₂O₂ concentrations were prepared and tested on freshly collected sperm for membrane integrity/viability by the trypan blue dye exclusion test. Only H₂O₂ concentrations that gave viability of $\geq 75\%$ on sperm were selected for the experiment. The experiment was carried out as follows: Phosphate buffered saline (PBS), and sperm differ depending on sperm concentration but generally between 4-6µl, 10 µl treatment solution (1%) and PBS to make a total of 1 ml, all mixed in Eppendorf® tubes and

incubated in a water bath at 32°C for 30 minutes. After incubation the Eppendorf® tubes were centrifuged at 3000 rpm for three minutes, then 900 µl of supernatant was discarded, the cell pellet was re-suspended and mixed with an equal volume of 1% low melting point agarose (Invitrogen: 15517-022) and layered on super frost slides. The layer was allowed to gel on an ice block. A third layer of 0.5% low melting point agarose was added on top and also allowed to gel on an ice block, the Comet assay was then carried out.

2.2.1.3.2.2 UVA irradiation of sperm cells

Three sets of 6 well cell culture plates (CLS 3516: Corning® Incorporated Co Star® NY, USA) were used for each experiment. The UVA radiation dose was provided using a lamp which consisted of two Waldmann F15/T8-PUVA tubes (Villingen-Scwenningen, Germany) in a casing (Wavelength range : 320-410 nm with peak emissions at 351 nm). In each set, a negative control with PBS in place of the chemicals and a positive control (80µM H₂O₂) were included for comparison. Each experiment was carried out in 3 independent repeats.

2.2.1.3.2.2.1 Treatment of sperm cells with ZnO and TiO₂

The amount of PBS used and the number of sperm varied depending on the sperm concentration, but on an average 4 µl of sperm, 986 µl PBS and 10 µl chemical were used. Cells were incubated at 32°C water bath. After incubation samples were centrifuged at 3000 rpm x 3 minutes.

2.2.1.3.2.2.2 Non-irradiation of sperm (D) A 6-well cell culture plate with PBS and cells was floated in a water bath in the dark maintained at 32°C for 30 minutes after which the chemical was added and further incubated in the dark maintained at 32°C for lymphocytes for another 30 min.

2.2.1.3.2.2.3 Pre-irradiation (PI) of sperm

The second 6-well cell culture plate with PBS and the cells was floated in a water bath maintained at 32°C and exposed to UV for 30 min (1.68 milliwatts/ cm²). Subsequently the chemical was added and incubated for another 30 min in the absence of UV at 32°C.

2.2.1.3.2.2.4 Simultaneous irradiation (SI) of sperm

The third 6 well cell culture plate with PBS and the sperm cells suspension were floated in the dark for 30 minutes at 32°C after which the chemicals were added and exposed to UV for 30 minutes (1.68 milliwatts/cm²) at 32°C, i.e. sperm received irradiation simultaneously with ZnO or TiO₂ treatment.

2.2.1.3.2.3 Slide preparation for the sperm assay

The primary preparation and agarose coating for superfrost slides was similar for both lymphocytes and sperm. After incubation of sperm cells with the different chemicals, the samples were centrifuged. An amount of 900 µl was then removed from the supernatant. The remaining 100 µl of the cell samples was mixed with 100 µl of 2% LMP. The second layer of the LMPA was laid over 2 duplicate slides and immediately covered with a cover glass (24 x 50 mm) and left to set on ice tray for 5 minutes. A third layer of 0.5% LMPA (100 µl) was applied on top of the second layer after carefully removing the cover slips and left to dry on ice for 5 minutes.

2.2.1.3.2.4 Lysis of sperm cells

The aim of the lysing step was to liberate the sperm DNA. After removal of the cover slips the slides were immersed laterally in cold lysing solution. The lysing solution

contained high salts to accelerate cell swelling and rupture the membrane. It consisted of 2.5M NaCl (BDH), 100 mM EDTA (Sigma: E 3154), 10 mM Trizma base (Sigma: T- 6066), 10% DMSO and 1% Triton X-100. The lysing solution was prepared during the incubation period, for each 50 ml (44.5 ml lysis solution, 0.5 ml Triton X-100 and 5 ml ddH₂O₂) were used. Two lysis steps were carried out to liberate the sperm DNA instead of one as for lymphocytes. The first lysis step started after the third layer of agarose solidified, the cover slips then removed and the slides were immersed in a cold lysis solution that contained 10 mM dithiothreitol (DTT) (Sigma: D-9779) and incubated in the refrigerator for 1 hour, thereafter, washed in PBS for 5 minutes. They were then immersed in a second lysis solution that contained 10 mg proteinase K (PK, Roche: 1000144) and incubated in the fridge for another hour, and washed in electrophoresis buffer for 5 minutes. DDT and PK were used to break the strong disulphide bonds in the sperm.

2.2.1.3.2.5 Sperm DNA unwinding and electrophoresis

The slides were removed from the lysing solution, then rinsed in an electrophoresis buffer PH ≥ 13 , and kept in an electrophoresis tank with electrophoresis buffer. Unwinding and electrophoresis was conducted at 4°C for 20 minutes each using a constant voltage of 25 volts and ~ 300 mA current.

2.2.1.3.2.6 Neutralization

The slides were removed from the electrophoresis buffer and neutralized with three washes of cold Tris buffer (pH 7.5) which contained Trizma base with the final concentration of 0.4 M. The aim of the neutralization step was to stop the alkaline buffer effect.

2.2.1.3.2.7 Slide staining and coding

In this assay ethidium bromide (EtBr) which is a DNA specific dye was used to visualize the slides at a concentration of (20 µg/ml); 60 µl were added to each slide. All slides were coded by an independent person to prevent bias in scoring.

2.2.2 Methods for genotoxicity and cytotoxicity of zinc oxide and titanium dioxide in HEp-2 cells

2.2.2.1 Cell culture of the HEp-2 cell line

Human negroid cervix carcinoma HEp-2 cells (HeLa derivative) were obtained from the European collection of cell cultures (ECACC, catalogue No 86030501). Such cells have been the mainstay of cancer research for determining the cytotoxic effects of different chemicals (Campbell et al., 2005; Cole et al., 1994; Kusumoto et al., 1988; Lebret et al., 2008). HEp-2 cells were maintained in continuous culture in pre-warmed Eagle's minimal essential medium in Earle's balanced salt solution (EMEM-EBSS) supplemented with 1% non-essential amino acids (NEAA), 2 mM glutamine, 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin and 100 mg/ml streptomycin (P/S) from Gibco, Paisley, UK. The medium was changed every 3-4 days and the cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were passaged upon confluence at a ratio of 1:3 stocks of cells were routinely frozen and stored in liquid nitrogen.

2.2.2.2 Chemicals

(Some of these chemicals have been described in Table 2.1, but are mentioned here again for ease of presentation). Zinc oxide nano-powder (ZnO; CAS 1314-13-2), and anatase titanium (IV) oxide nano-powder (TiO₂, 99.7%, CAS 1317-70-0), low melting-

point agarose (LMA), normal melting-point agarose (NMA), thiazolyl blue tetrazolium bromide [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye (CAS 298-93-1), ; neutral red (NRU) dye (CAS 553-24-2); ethidium bromide (EtBr) and Triton X-100 were purchased from Sigma Aldrich, Poole, Dorset, UK. Phosphate buffered saline (Ca^{2+} , Mg^{2+} -free; PBS), Trypan blue dye solution and 10,000 U/ml of trypsin–EDTA were purchased from Gibco. Acrylamide / bis-acrylamide solution (30%), Bio-Rad protein assay dye reagent concentrate (CAS 500-0006), buffers, membranes and films were purchased from Bio-Rad, Hertfordshire, UK. Phosphotyrosine mouse monoclonal antibody (P-Tyr-100) and anti-mouse IgG-HRP linked antibody were purchased from Cell Signalling Technology, Hitchin, Hertfordshire, UK.

2.2.2.3 Particle preparation and characterization

Nanoparticles were suspended in 10 ml EMEM-EBBS medium at concentrations of 10, 20, 50, and 100 $\mu\text{g/ml}$. Suspensions were probe-sonicated at a current of 30 W for 5 minutes on and off then allowed to equilibrate for different times: 0, 2, 4, 24, and 48 hours. The first two times were chosen to look at more immediate effects, the latter two time points to evaluate long-term effects. The resulting ZnO and TiO_2 suspensions were measured as a function of incubation time. Prior to evaluation of the toxic potential, the mean size (hydrodynamic diameter) of the nanoparticles was determined using a high performance particle sizer (Malvern Instruments Ltd., Worcestershire, U.K.). Before analysis suspensions were briefly shaken to resemble the cell culture incubation conditions. Suspensions were placed in disposable cuvettes and three consecutive measurements at 25 °C each consisting of three runs were undertaken.

2.2.2.4 Cytotoxicity assays

2.2.2.4.1 MTT assay

The MTT assay to evaluate the mitochondrial activity was undertaken according to the originally described method (Mosmann, 1983). HEp-2 cells were plated in 96-well plates and the cell concentration ($\sim 1.5 \times 10^4$) was chosen as previously successfully used (Khan et al., 2007; Uboldi et al., 2009). The cells were incubated for 24 hours at 37° C and 5 % CO₂, then incubated for different time periods (2, 4, 24 and 48 hours) with ZnO or TiO₂ nanoparticles. The MTT dye was added 4 h prior to completion of incubation periods. The medium from each well was discarded and the resulting formazan crystals were solubilized by adding 200 µl of dimethylsulphoxide (DMSO) and quantified by measuring absorbance at 570 nm.

2.2.2.4.2 Neutral red uptake (NRU) assay

The NRU assay was performed to determine the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells (Borenfreund and Puerner, 1985). The medium was discarded and 100 µl of neutral red dye (50 µg/ml) dissolved in serum free medium was added to each well. After incubation at 37 °C for 3 h, cells were washed with PBS and the dye taken up by cells was dissolved in 200 µl of a fixative solution (50% ethanol, 49% ddH₂O₂ and 1% acetic acid) and added to each well. Absorbance was taken at 570 nm in MRX 11 micro-plate reader (Dynex Technologies, USA), using the software Revelation version 4.02.

2.2.2.5 Comet assay on HEp-2 cells

2.2.2.5.1 Cell viability

Prior to the Comet assay, cells were incubated with different concentrations (10, 20, 50, 100 µg/ml) of ZnO or TiO₂ nanoparticles for 4 hours then assayed for viability using the Trypan blue dye exclusion test. The cut-off point was 75% as suggested by Henderson and colleagues (Henderson et al., 1998).

2.2.2.5 Comet assay on HEp-2 cells

The sub-confluent monolayer was exposed to four different concentrations of ZnO and TiO₂, and 2.72 µg/ml (= 80 µM) of hydrogen peroxide (H₂O₂) as a positive control. There was also a negative control using untreated cells. After an incubation of 4 hours, cells were washed with cold PBS and harvested with trypsin-EDTA and followed by centrifugation at ~180 g for 9 minutes. The pellet was finally resuspended in PBS. The Comet slides were prepared by the method as previously described (Tice et al., 2000). The cell suspension (100 µl) was mixed with 100 µl of 1% LMA, of which 100 µl was spread onto microscope slides pre-coated with 1% NMA. The slides were kept overnight at 4 °C in freshly prepared, chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, with 1% Triton X-100; pH 10). The slides were subjected to freshly prepared cold alkaline electrophoresis buffer (1 mM EDTA sodium salt and 300 mM NaOH; pH ≥13) for DNA unwinding, then electrophorized for 30 minutes each at 4 °C in electrophoresis buffer. The electrophoresis buffer was neutralized with Tris buffer (400 mM, pH 7.4) and stained with 20 µg/ml ethidium bromide. Fifty cells from each concentration were scored 'blindly' at a final magnification of 400x using an image analysis system (Komet 4.0 attached to a fluorescence microscope equipped with a CCD camera). The Olive tail moment and % tail DNA were used as parameters to measure the DNA damage in each cell. Three repeat experiments were carried out over the dose ranges used.

2.2.2.6 Cytokinesis-block micronucleus (CBMN) assay on HEp-2 cells

The *in vitro* CBMN assay was performed as described previously (Fenech, 1993). HEp-2 cells were grown in 6-well cell culture plates suspended in 2 ml EMEM medium at a concentration of 1×10^5 . After 24 hours of culture, either the TiO₂ nanoparticles (10, 20 and 50 µg/ml) or ZnO nanoparticles (10, 20, 50 and 100 µg/ml) or mitomycin C (positive control; 0.4 µM = 0.134 µg/ml) were added to the cultures. An untreated culture served as the negative control. Following 2 hours of incubation, the culture medium was removed and the cells were washed with PBS. After the treatment, 7.5 µl cytochalasin B (final concentration 0.01 mg/ml) was added to fresh culture medium and incubated overnight. HEp-2 cells were trypsinized and centrifuged at ~180 g for 8 minutes. The cell pellet was treated with cold hypotonic solution (70 mM KC1) for 15 minutes and centrifuged at ~180 g rpm for 8 minutes, fixed in methanol / acetic acid (3:1), and air-dried. Slides were stained with 5% Giemsa for 10 min for the detection of micronuclei in binucleated cells. Micronuclei were scored and frequencies were evaluated per 1000 binucleated cells.

2.2.2.7 Detection of tyrosine phosphorylation in HEp-2 cells

2.2.2.7.1 Culture of HEp-2 cells

The cells were maintained in continuous culture in pre-warmed Eagle's minimal essential medium in Earle's balanced salt solution (EMEM-EBSS) supplemented with 1% non-essential amino acids (NEAA), 2 mM glutamine, 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (P/S) from Gibco, Paisley, UK. The medium was changed every 3-4 days and the cells were maintained in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were passaged upon confluence at a ratio of 1:3. Stocks of cells were routinely frozen and stored in liquid nitrogen.

2.2.2.7.2 Treatment of HEp -2 cells and protein extraction

A sub-confluent HEp-2 monolayer was treated with different concentrations (0, 10, 20, 50 and 100 $\mu\text{g/ml}$) of ZnO or TiO₂ nanoparticles and incubated for 4 hours. Untreated cells served as a negative control. After the treatment, the cells were trypsinized and suspended in 10 ml PBS and centrifuged at $\sim 180\text{ g}$ for 9 minutes. The HEp-2 cell pellet was resuspended in 200 μl PBS and assessed (10 μl) for viability and membrane integrity using the Trypan blue dye exclusion test. Ninety microliters of cell suspension were added to 10 ml PBS for Western Blotting, while the rest (100 μl) was used for the Comet assay. After centrifugation ($\sim 180\text{ g}$ for 9 minutes), RIPA lysing buffer in addition to protease and phosphatase inhibitors was added to the pellet. RIPA was used because its contents (table 2.4) are potent protein solubilizers without protein degradation. The resulting cell suspension was briefly sonicated (~ 5 seconds) at 30 W. Protein concentrations were determined in each sample using the Bio-Rad microplate *DC* assay (Lowry). SDS detergent was used to break up cell membranes to release proteins for later analysis using Western blotting. The cell concentration in the lysing buffer was 10^4 cells / μl of lysis buffer. Samples were then collected in Eppendorf® tubes, labelled and kept at -20°C . A hole was made in the cap of each tube to prevent popping during boiling. The cell lysate was suspended in Laemli sample buffer and 35 μg proteins from each sample was loaded on SDS gel and detected by the immunoblotting technique using a phosphotyrosine mouse monoclonal primary antibody (P-Tyr-100) and an anti-mouse IgG-HRP linked secondary antibody.

Table 2.4 Protein extraction and Western blotting buffers

Buffer	Constituents
Blotting buffer (10X)	0.25 M TRIS and 1.92 M glycine. On use add 20% methanol
Laemmli sample buffer 2x concentrate	4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.125 M Tris HCl, pH 6.8, and 0.004% bromphenol blue
Resolving gel buffer	1.5M tris HCl buffer pH 6.8
RIPA buffer	150 mM NaCl, 0.5% sodium deoxycholate, 1.0% IGEPAL, 0.1% SDS and 50 mM Tris, pH 8.0
SDS buffer(10X)	0.25 M Tris base, 1.92 M Glycine and 0.1% SDS pH 8.5
Stacking gel buffer	0.5 M Tris HCl buffer pH 8.8

2.2.2.7.3 Protein concentration

The protein concentration was measured using Bio-Rad *DC* Protein microplate assay which is colorimetric assay based on Lowry assay (Lowry et al., 1951). The Bio-Rad *DC* Protein kit consists of reagent A which is an alkaline copper tartrate, reagent B

which is folin reagent and reagent S to activate reagent A. The assay is based on the following reaction:

Alkaline copper tartrate + Proteins \longrightarrow loss of O_2 ions from folin \longrightarrow reduced Folin products \longrightarrow development of blue colour with maximum absorbance at 750. The colour is basically due to amino acids tyrosine and tryptophan in different proteins.

The microplate assay protocol was performed as follows:

1. Reagent A' which is the active form of reagent A was prepared by adding 20 μ l of reagent S to reagent A
2. Eight protein standards from 0- 2 mg/ml in RIPA buffer were prepared.
3. In 96 wells plate 5 μ l of standards and samples were pipetted in triplicate
4. 25 μ l of reagent A' were then added into each well.
5. Reagent B (200 μ l) was then added to each well.
6. Reagents were then mixed through gentle agitation and left to set for 15 minutes.
7. The plate was taken to a microplate reader and the absorbances were read at 750 nm

2.2.2.7.4 Western blotting

2.2.2.7.4.1 Sodium dodecyl sulphate-polyacrylamide gel (SDS- PAGE)

Tris buffers (pH 6.8 & pH 8.8) were prepared for the resolving and stacking gels (table 2.4). The catalysts APS and TEMED were added for poly-acrylamide gel polymerization.

At the bottom a resolving gel with a pH of 8.8 was poured between 2 glass plates to a level of 1 cm below the teeth on the comb and overlaid with 0.1% (w/v) SDS solution. The overlay was poured off after the gel had set. 4% (w/v) stacking gel solution with a pH 6.8 was then poured on top of the solidified separating gel to pack proteins in together after loading. While gels were setting, the protein samples were prepared by

mixing each sample with the 2X sample buffer (1:1) then heated in boiling water for 5 min. The Laemli buffer was used because it contains 2-mercaptoethanol that reduces the disulfide bonds. It contains SDS detergent which denatures the proteins and gives each protein a negative charge, accordingly each protein separates on a size base and not on a charge base. In addition, bromophenol blue is used because of its colour that makes it easier see the samples and glycerol to increase samples viscosity. Samples were then centrifuged for 10 sec after boiling, and loaded into separate lanes with the first left lane kept for the molecular weight reference. The polyacrylamide gel was run using a discontinuous system (Laemmli, 1970). A mini Protean-11 gel apparatus (Bio-Rad, Hertfordshire, UK) was used to run all gels. The gel was run initially slowly through the stacking gel (50 Volts), to give sharper bands, then run at 100V (constant voltage) throughout the resolving gel. The protein ladder and the dye front was watched to determine when to stop the gel. The gels were immersed in electrophoresis buffer (25 mM Tris base, 192 mM glycine and 0.1% (w/v) SDS) within the running tank and 20µl of protein was loaded per well. On each gel, one lane contained 10µl of a biotinylated protein ladder of molecular weight standards (cell signalling). The gel ran for ~ 2 hours at a constant voltage of 100V. The SDS gel was used to separate proteins according to their size by electric current. Low percentage gels separate larger proteins whereas higher percentage gels separate smaller proteins better. The problem is that all proteins have a charge associated with them, and in an electrical current this could cause problems. That was solved by the addition of SDS to the protein samples. SDS binds to proteins every few amino acids and neutralizes the charge differences that proteins have. This allows proteins to be separated by size and not by charge. Every sample was loaded carefully and slowly to prevent the sample leaking out of the lane.

2.2.2.7.4.2 Transfer to membrane: After electrophoresis, proteins were transferred to blotting membrane using a Bio-Rad electrophoresis transfer unit by the method of (Towbin et al., 1979). Transfer was carried out in blotting buffer (25 mM Tris base, 192 mM glycine) in addition to 20% (v/v) methanol for one hour at a constant voltage of 100.

2.2.2.7.4.3 Blocking: After transfer the nitrocellulose membranes were incubated with a generic protein to bind to any remaining sticky places on the membrane. The blocking solution contained 5% (w/v) BSA in Tris buffered saline containing Tween 20 “TBS-T” (150 mM NaCl, 20 mM Tris base and 0.1% (v/v) Tween 20, pH 7.4) for one hour at room temperature with gentle shaking. Membranes were washed 3 X for 10 minutes with TBS-T.

2.2.2.7.4.4 Primary antibody: A primary antibody was then added to the solution which was able to bind to its specific protein. The blotting membranes were incubated over night at 4°C with shaking with the primary antibody mouse immunoglobulin IgG1 phosphor-Tyrosine Mouse mAb (P-Tyr-100) (Cell Signalling Technology) and GAPDH mouse monoclonal primary antibody was used as a loading control (ab9484). A monoclonal antibody was used because it gives a better signal and lower background and has high specificity. The primary antibody was diluted 1:2000 in TBS-T containing 5% (w/v) BSA. Membranes were then washed 3 X for 10 minutes with TBST.

2.2.2.7.4.5 Secondary antibody: A secondary antibody-enzyme conjugates “Anti-mouse IgG, HRP-linked antibody”, recognizing the primary antibody, was added to find locations where the primary antibody bound. The secondary antibody was diluted 1:3000 with 5% (w/v) non fat milk in TBS-T solution together with HRP- conjugated anti- biotin antibody diluted (1:1000) to detect biotinylated protein markers. The

membrane was incubated at room temperature with gentle agitation for one hour. The membrane was washed 4 times for 15 minutes each.

2.2.2.7.4.6 Detection: The membrane was stained with enhanced chemiluminescence (ECL) solutions 1 and 2. The membrane was exposed to Kodak X ray film for 2 minutes. The film was immediately developed through quick and consecutive processes by immersing it in the developing solution, washing in water and lastly fixative solution.

2.2.3 Methods: A comparison of the effects of nanoparticles in peripheral blood lymphocytes from respiratory disease patients and healthy individuals using genotoxic endpoints and ras p21

2.2.3.1 Materials and Methods

For materials used in each experiment refer to table 2.1

2.2.3.2 Experimental design

The experimental design was detailed in chapter 5 section 5.2.2.1.

In general, the experiment was designed to find if there are differences in response between three different groups of respiratory diseases patients (lung cancer, chronic obstructive pulmonary disease (COPD), asthma) and a healthy control group lymphocytes exposed to different concentrations of TiO₂ NPs using different end-points for genotoxic effects that include the Comet assay, CBMN assay and ras oncoprotein level determinations.

2.2.3.3 Blood collection and lymphocyte isolation

In general, 36 ml of blood were taken from each patient or healthy control in a heparanized tubes (9 ml in each of 4 tubes), all samples processed (fresh) within 2 hours of isolation. Lymphocytes were then isolated as described in section 2.2.1.2.2 which was later used in the Comet assay and lymphocyte cell culture for ras P21 detection.

2.2.3.4 Nanoparticles and treatments preparation

Prior to evaluation of the toxic potential of TiO₂, different concentrations of TiO₂ suspensions (5, 10, 30 and 50 µg/ml) were prepared in 10 ml RPMI 1640 medium through probe-sonication at current 30 W. The different TiO₂ suspensions particle sizes were determined using a high performance nano sizer (Malvern Instruments Ltd., Worcestershire, U.K.). Different TiO₂ concentrations were then prepared (10, 30 and 50 µg/ml) for the Comet and ras p21 detection assays. For the CBMN 5 and 10 µg/ml of TiO₂ concentrations were prepared. In addition 80 µM of hydrogen peroxide (H₂O₂) and 0.4 µM mitomycin C (MMC) were prepared as positive controls for the Comet and CBMN assays respectively.

2.2.3.5 Cell viability and Comet assay

The lymphocytes were treated for 30 minutes at 37°C with the different concentrations of all chemicals used. For TiO₂, 10, 30 and 50 µg/ml were used, for H₂O₂, 80 µM was used and for MM C 0.4 µM. Thereafter, cell viabilities were assessed by a trypan blue exclusion test as detailed in section 2.2.1.3.1. Generally, the detection of DNA damage in the respiratory disease patients and healthy controls in the Comet assay pH ≥ 13 system was carried out with different TiO₂ concentrations (10, 30 and 50 µg/ml) and H₂O₂ (80 µM) as a positive control and a negative control of untreated lymphocytes. The Comet assay was carried out as detailed in section (2.2.1.3).

2.2.3.6 Cytokinesis-block micronucleus assay (CBMN)

Peripheral blood is the most frequent source of human lymphocytes. Human lymphocytes have two major functions cellular immunity through T lymphocytes which are thymus- dependent cells and antibody production through B lymphocytes. Healthy individuals have 70% T- lymphocytes of the total lymphocyte cells. Normally, foreign

antigens provoke immune response with cellular division and proliferation. Mitogens are substances mimicking foreign antigens that transform the majority of lymphocytes into a mitotically active state. Phytohaemagglutinin (PHA) is a mucoprotein and widely used as a mitogen. Phytohaemagglutinin occurs naturally in red kidney bean (*Phaseolus vulgaris*). Hence, mitogen-stimulated lymphocytes by PHA, proliferate and divide with subsequent stimulation of further cell type divisions. The whole process occurs as an immune response (Cantrell et al., 1993).

Normally lymphocytes stimulated with PHA, start dividing during the first 24 hours post exposure that occurs without clear morphological transformation.

The (CBMN) assay is used to assess chromosome damage and can detect aneuploidy or chromosome loss besides clastogenicity or chromosomal breakage.

The advantages of CBMN assay can be summarized in the following:

- It is relatively simple, inexpensive, and rapid, has a cell to cell approach, can detect chromosome mutations, apoptosis and necrosis and is applicable to variety of cell types. In combination with fluorescence in situ hybridization (FISH) it can discriminate between clastogens and aneugens. In addition it could be used as a useful biomarker to access cancer risk.
- Also can be used as an endpoint to assess the identification of chromosome mutations. It also can show cells that go nuclear division and that did not divide and can assess cell proliferation (% binucleated cells).

The CBMN has some disadvantages that can be summarized in the following:

- It requires cell division for expression of MN.

- It does not detect all structural chromosome aberrations (only acentric fragments).
- The cytochalasin B itself could be cytotoxic and its cytotoxicity differs between cell types.

The scoring criteria for micronuclei selected according to Fenech et al, 2000:

- Binucleated cells with main nuclei of approximately equal size that separate, touch, are linked by nucleoplasmic bridges or even overlap as long as there are clear boundaries between the 2 nuclei.
- Binucleated cells in which the main nuclei are undergoing apoptosis are not scored because MN may be caused by the apoptotic process
- The MN diameter should be less than 1/3 of the main nucleus
- There should be a clear identification of the nuclear boundary between the main nucleus and the MN which should be separated from or slightly overlap with the main nucleus.
- Staining of MN should be similar to the main nucleus

2.2.3.6.1 Blood cultures

Blood cultures were prepared from each (coded) sample in 25 cm³ cell culture flasks, each culture consisted of 500 µl of whole blood added to 4.5 ml fresh medium of RPMI 1640 with Glutamax-I which contained 20% fetal bovine serum (Gibco) and 1% penicillin-streptomycin solution (P/S), then fresh medium allowed the cells to grow. 130 µl of phyohaemagglutinin (PHA), a mitogen, was added to reach a final concentration of 2.5%, added because lymphocytes need to be stimulated to divide and proliferate. Cultures were then left in an incubator whilst the chemicals 50 µl of TiO₂ at different concentrations (5 and 10µg/ml) and 50 µl of MMC were added as a positive control to

reach a final concentration of 0.4 μM (Meynard et al., 2007), a negative control of untreated lymphocytes was as well prepared. At time 24 hours, Cytochalasin B at a final concentration of 6 $\mu\text{g/ml}$ was added to cell cultures at 44 hours which prevented the cells undergoing cytokinesis, so that binucleated cells were formed. Incubation is continued until 72 hours. Each sample was treated in duplicate flasks with TiO_2 as well as positive and negative controls (Fenech, 2007).

2.2.3.6.2 Hypotonic treatment and fixation

The lymphocyte cells were harvested by centrifugation at 900 rpm (200g) for 9 minutes then treated with hypotonic treatment for ~5 minutes, centrifuged again for another 9 minutes at 900 rpm (200g) then fixed in 3:1 v/v methanol: acetic acid and 3 drops of formaldehyde. The fixation step was repeated until the pellet was clear.

2.2.3.6.3 Slide preparation

Two slides were prepared from each fixed cell sample in a fixative (concentration depending on the pellet). A total of 4 slides per duplicate sample were prepared. Each slide was dropped with 2 drops each x 20 μl of cell suspension then left to air dry. The slides were stained with 10% Giemsa stain in phosphate buffer for 10 minutes, left to air dry over night, mounted and covered with a cover slip.

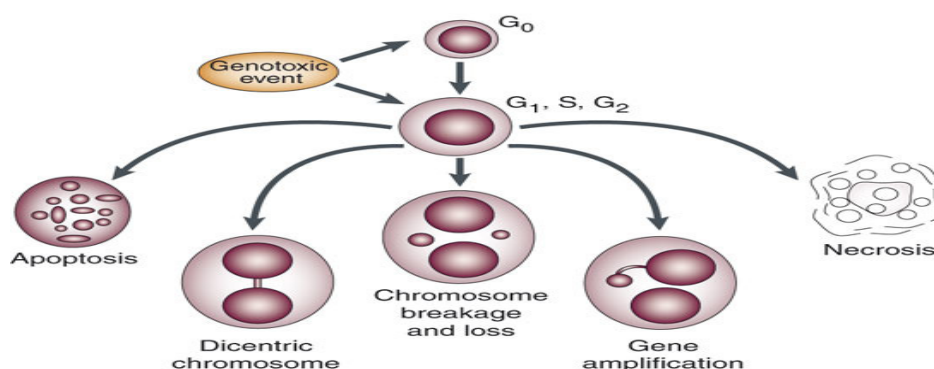


Figure 2.1 The various possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents
From an article: Cytokinesis-block micronucleus cytome assay, by Michael Fenech *Nature Protocols* **2**, 1084 - 1104 (2007)

2.2.3.6.4 Slide scoring

A total of 31 patients (13 lung cancer, 10 COPD and 8 Asthma) and 15 controls were used in the CBMN assay; 5 samples were excluded for technical reasons.

The slides were coded and scored according to Fenech criteria (Fenech et al., 2000). The Micronuclei were counted using a 100X oil immersion objective on a light microscope and X12.5 eye piece. One thousand cells with clear cytoplasm were scored to find out the number of the Mono, Bi and multinucleated cells which later used to calculate the nuclear division index (NDI) and the % BiNCs. One thousand BiNCs that fit Fenech criteria were then scored simultaneously with the number of the MN, nucleoplasmic bridges NPBs, nuclear buds, mono-nucleated cells with MN and multi-nucleated cells with MN.

2.2.3.7 Ras oncoprotein detection technique

2.2.3.7.1 Lymphocyte cell culture and chemical treatment

The population for ras p21 study was detailed in table 5.2. Twelve ml of blood from each patient or control was used to isolate 4 separate lymphocyte pellets. The pellet of freshly isolated lymphocytes (lymphocytes isolation technique detailed in section 2.2.1.2), was resuspended in 4.5 ml fresh medium of RPMI 1640 with Glutamax-I which contains 20% fetal bovine serum and 1% penicillin-streptomycin solution (P/S) and 130 µl of PHA to reach a final concentration of 2.5%. Four separate lymphocyte cultures were prepared for each individual. Cultures then incubated in a humidified incubator supplemented with 5% CO₂ at 37°C for 48 hours. Thirty minutes before the

end the incubation period 50 µl of TiO₂ different concentrations (10, 30 and 50µg/ml) were added to the cell cultures, untreated lymphocytes culture left as a negative control, cultures were then incubated for further 30 minutes. At the end of the 30 minutes incubation, cultures were placed in 50 ml falcon tubes then centrifuged at 900 rpm (200g) for 9 minutes, the supernatant then discarded and the pellet then washed twice in PBS by centrifugation at 4°C. After the last centrifugation the pellet was then suspended in 100 µl of RIPA buffer, protease inhibitor cocktail was added as well and the cells left to lyse for 30 minutes on ice, thereafter centrifuged at 13000 rpm for 20 minutes (X 400g) at 4°C, supernatant was then moved to another labelled tubes, 5 µl from each sample was taken for DC Bio-Rad protein assay as described in section (2.2.7.3) and the rest of the cell lysates stored at -80°C until used in ras p21 immunoblotting assay.

2.2.3.7.2 Western blotting for ras p21 detection

The Western blotting technique in section (2.2.7.4) was used for ras P21 detection, the only exceptions are the primary antibody was pan ras p21 mouse monoclonal antibody (ab86696) from Abcam, and SW480 cell lysate (Abcam) was used as a positive control (Bocca et al., 2010; Min et al., 2010)

2.2.4 Statistical analysis

In all experiments the results obtained from treated cells (lymphocytes, sperm and Hep-2 cells) with either ZnO, TiO₂, H₂O₂ or MMC were compared to the negative control of untreated cells to find the *p* value and different statistical methods were used in this study depending on the result of the variance normality of distribution and /or homogeneity which were checked by Kolmogorov-smirnov method.

In case of Comet assay, slides were scored at 540 nm filter wave length using (Leica DMLB) fluorescence microscope connected to a Hitachi KPMI/EK charge coupled device (CCD) monochrome camera connected to a computer with a Komet 4.0 programme (Kinetic Imaging, Liverpool, UK). This was used to measure Comet parameters. The images from each cell were received by a camera then went through projection lens and appeared in a computer monitor screen at a magnification of 200X resulting from 20X objective lens and 10 X eye piece gave a magnification to score the slides.

The number of cells per sample scored varies. In the *in vivo* experiments which have a lower sensitivity due to internal variations, up to 150 cells in 3 repeats have been scored blindly in coded slides (50 cells obtained from duplicate slides per dose or culture) as recommended (Ambrosini et al., 2006; Tice et al., 2000). In studies where clinical samples were used each group was accessed as one unit due to limited access to clinical samples 2 independent experiments were carried out and 100 cells were scored from duplicate slides.

The Olive tail moment which “is the value of the tail density multiplied by the migration distance (tail length)” (Olive et al., 1991) was automatically generated by the computer and used for lymphocyte assays. The Olive tail moment is considered as a preferred parameter in describing the observed DNA damage as it is more uniform. However, for sperm due to baseline damage of 20% SSB, head DNA was used.

For clinical samples the Olive tail moment (OTM) and % tail DNA parameters were used as the use of two different parameters have been recently recommended (Kumaravel et al., 2009). Kolgomrov-Smirnov used to test the OTM and % tail DNA data for normality of distribution, the data have been found to have normal distribution,

accordingly the parametric ANOVA test was applied. Groups were compared in 2 different ways, in the first each group was compared to its counterpart untreated control and in the second each patient group or confounder was compared to its parallel healthy control sample.

The data obtained was tested for normality of distribution using the Kolmorov- Smirnov test. Once the data show normal distribution ANOVA test was used, and when the data did not show normal distribution (nonparametric) Mann- Whitney U test was used on median values. However, for a clearer comparative presentation, histograms have been produced of 3 repeat experiments showing means and standard errors of the means.

For MTT and NRU assays, 4 wells of a micro-plate were used and each was seeded with 1.5×10^3 HEp-2 cells and the means of 3 repeat experiments were analysed using Microsoft Excel 2007 and the software for Windows SPSS 15.0.

For CBMN assays because the data was nominal the number of MN in 1000 binucleated cells was compared between the untreated and treated for each sample using the graph pad 2x2 contingency (Fisher exact test) for the p value. For the nuclear division index (NDI) which can be used as an indicator of the cytostatic effect of the chemicals used, it was calculated according to the formula: $NDI = (M_1 + 2M_2 + 3M_3) / N$; where M_1 , M_2 and M_3 presented mononucleated, binucleated and multinucleated cell respectively while N represented the total viable cells scored or with the Chi square (χ^2).

The null hypothesis theory which assumed no significant differences or no treatment effect at a fixed level of 0.05 was used to determine the level of significance. Accordingly, the p value was determined for the extreme values as < 0.05 and

represented as (*) when the null hypothesis was rejected at the level of 5% and represented as (**) if the null hypothesis rejected at the level of 1% and the *p* value was said to be significant at 0.01 and (***) were used if *p* was < 0.001.

In all statistical work that was carried out, Microsoft EXCEL 2007 and SPSS 15.0 were used. For a clearer comparative presentation, tables and histograms have been produced showing means of the means and means of the standard errors of the means.

Chapter 3

The effect of zinc oxide and titanium dioxide nanoparticles in the Comet assay with UVA irradiation of human sperm and lymphocytes

3.1 Introduction

The use of nanotechnology has seen a surge in recent years and the increasing applications of nanomaterials in diverse fields such as biotechnology, life sciences, medicine, defence and engineering have raised awareness of potential genotoxicity. These particles, by virtue of their size could enter the body either orally, by inhalation, absorption or could be injected into the body during medical procedures. Hence, caution should be exercised during the use and disposal of such manufactured nanomaterials to prevent unintended toxic effects on organisms and undesirable environmental impacts. The unique physico-chemical properties of these particles could produce unpredictable effects and the high surface to volume ratio could result in greater reactivity.

Inhalation studies with rats exposed to ultra-fine TiO₂ aggregate and carbon black showed that both compounds induced lung tumours in rats at considerably lower gravimetric lung burdens than larger sized TiO₂ particles (Oberdorster, 1996). Significant species differences have been reported in the pulmonary responses to inhaled TiO₂ particles. For example, rats developed a more severe and persistent pulmonary inflammatory response than mice or hamsters and were also unique in the development of progressive fibro-proliferative lesions and alveolar epithelial metaplasia

in response to exposure to a high concentration of TiO₂ particles. Published studies in CHO cells showed ZnO to be clastogenic, but may not represent a photo-genotoxic effect when compared to 8-MOP (Dufour et al., 2006). TiO₂ also did not exhibit photochemical genotoxicity in the same system (Theogaraj et al., 2007). Nagakawa *et al.*, (1998) demonstrated photo-genotoxic effects with TiO₂ particles in mouse lymphoma (L5178Y) cells in the Comet assay and clastogenicity in Chinese hamster lung cells, but positives were generally only at toxic doses.

Up to now, hardly any studies have focused on the effect of nanoparticles on the germ cells. One study has reported that when gold nano particles are mixed with human spermatozoa, penetration of gold nanoparticle into the sperm heads and tails was observed and 25% of them lost their motility. This led the authors to conclude that gold nanoparticles had spermatotoxic effects (Wiwanitkit et al., 2009). The effects of different types of nanoparticles on mouse spermatogonial germline stem cells also demonstrated a concentration-dependent toxicity for silver, molybdenum and aluminium nanoparticles tested, whereas the corresponding soluble salts had no significant effect (Braydich-Stolle et al., 2005). In spite of the fact that ZnO and TiO₂ are widely used ingredients in dermatological preparations and sunscreens and many other products, only two of the investigations have focused on the effects of these nanoparticles on human germ cells. DNA damage to somatic cells can give rise to cancer but damage to germ cells can not only give rise to cancer, but also cause congenital defects in future generations. Accordingly, it was decided to investigate the effect of commonly used ZnO and TiO₂ (anatase form) nanoparticles in human sperm and lymphocytes *in vitro* using the Comet assay, a simple but sensitive technique for evaluating intercellular differences in DNA damage (Singh et al., 1988; Tice et al., 2000). Sperm and lymphocytes represent primary human cells, with some metabolic

activities intact, of a haploid and diploid nature respectively. It is very useful to obtain information on cells of direct relevance to man. For over 40 years human lymphocytes have been used as surrogates for prediction of toxic effects in man (Albertini et al., 2000).

3.2 Materials and methods

3.2.1 Preparation of ZnO and TiO₂ nanoparticle stock suspensions

Refer to methods (2.2.1.1), ZnO/TiO₂ (anatase) nano-powders were suspended in DMSO then the optical density (OD) was measured. The suspensions were then filtered through a 0.2 µm syringe filter and the OD of the filtrate measured. Using the OD before and after filtration, the concentrations of the filtrate was determined. Serial dilutions of the filtrate were made to prepare the stock suspensions to be used for the experiments. Four concentrations were used for each of the compounds tested. For ZnO, the concentrations used were 11.5, 46.2, 69.4 and 92.3 µg/ml and for TiO₂ the doses used were 3.73, 14.92, 29.85 and 59.7 µg/ml.

3.2.2 Ethics: The collection of lymphocytes and sperm had been approved by the University of Bradford Subcommittee for ethics in research involving human subjects (Reference 0405/8).

3.2.3 Cell isolation: The isolation of lymphocytes was described in chapter 2 section 2.2.1.2.1 and 2.2.1.1.7. For the semen sample collection refers to section 2.2.1.3.2.

3.2.4 UV Irradiation: Three sets of 6 well cell culture plates were used for each experiment (details on chapter 2 sections, 2.2.1.3.1.3 and 2.2.1.3.2.2).

3.2.4.1 D method: Refer to methods 2.2.1.3.1.3.1 and 2.2.1.3.2.2.2.

3.2.4.2 PI method: Refer to method 2.2.1.3.1.3.2 and 2.2.1.3.2.2.3.

3.2.4.3. SI method: Refer to method 2.2.1.3.1.3.3 and 2.2.1.3.2.2.4.

3.2.5 The Comet assay

The Comet assay carried out as described in chapter 2 sections 2.2.1.3.1 and 2.2.1.3.2. The main experiments were repeated three times making a total of 150 cells per experimental observation.

3.2.6 Statistical analysis

The data obtained were tested for normality of distribution using the Kolmorov-Smirnov test and found to violate the normal distribution pattern. The non-parametric Mann-Whitney U test was used to compare treated samples with the negative control values to obtain the p values. However, for a clearer comparative presentation, histograms have been produced of the combined results from triplicate experiments, showing the means and standard errors.

3.3 Results

3.3.1 Effect of H₂O₂ in human lymphocytes and sperm

The Comet assay was carried out as described in chapter 2 sections 2.2.1.3.1.2 and 2.2.1.3.2.1.

The dose response curve of human lymphocytes and sperm in the Comet assay, after treatment with H₂O₂ and incubation for 30 minutes. A total of 150 cells were scored for each dose in three independent experiments. The Kolmogorov-Smirnov test (KS-test) was used to test the normality of the sample distribution. The average median of the Olive tail moment (lymphocytes) or % head DNA (sperm) of the control (cells treated with PBS) was compared versus the treatment doses with Mann-Whitney. The bars indicate the standard errors for three independent experiment (ns, not significant, ** $p < 0.01$, *** $p < 0.001$).

3.3.1 DNA damage on sperm cells treated with H₂O₂

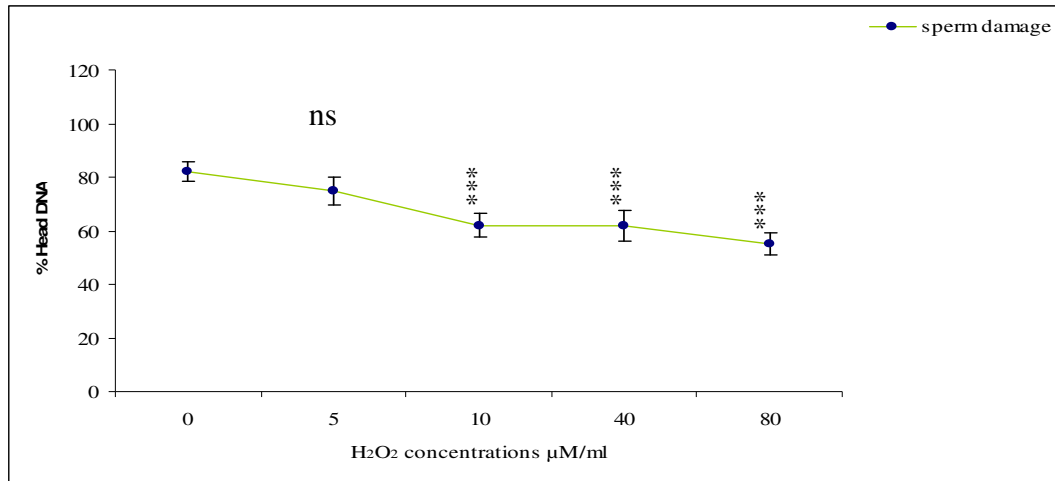


Figure 3.1 The dose response curve of the effect of different H₂O₂ concentrations on human sperm in the Comet assay, in addition to a negative control of untreated sperm cells. The means of the % head DNA of untreated sperm (control) compared to the means of treated sperm cells with one way ANOVA using SPSS 15. Line indicate mean % head DNA of 3 experiments ns indicates and *** $p < 0.001$. Bars indicate standard errors.

3.2 DNA damage and repair in lymphocytes treated with H₂O₂

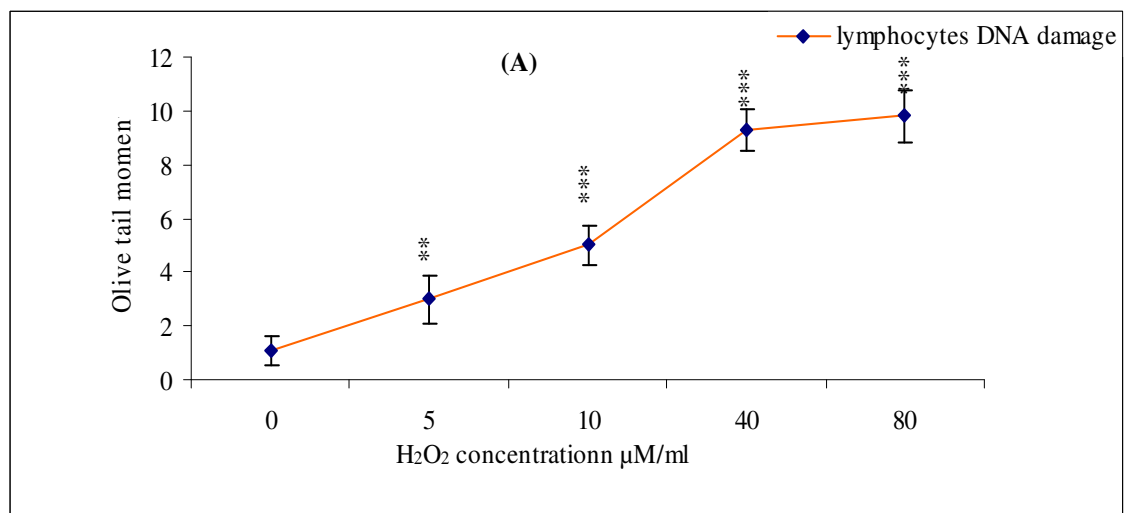


Figure 3.2A The dose response curve of the effect of different H₂O₂ concentrations, in addition to a negative control of untreated lymphocytes, in human lymphocytes in the Comet assay. The means of the OTM of untreated lymphocytes (control) compared with the OTM means of treated lymphocytes analysed by one way ANOVA using SPSS 15. Line indicate mean OTM of 3 experiments ** $p > 0.01$ and *** $p < 0.001$. Bars indicate standard errors.

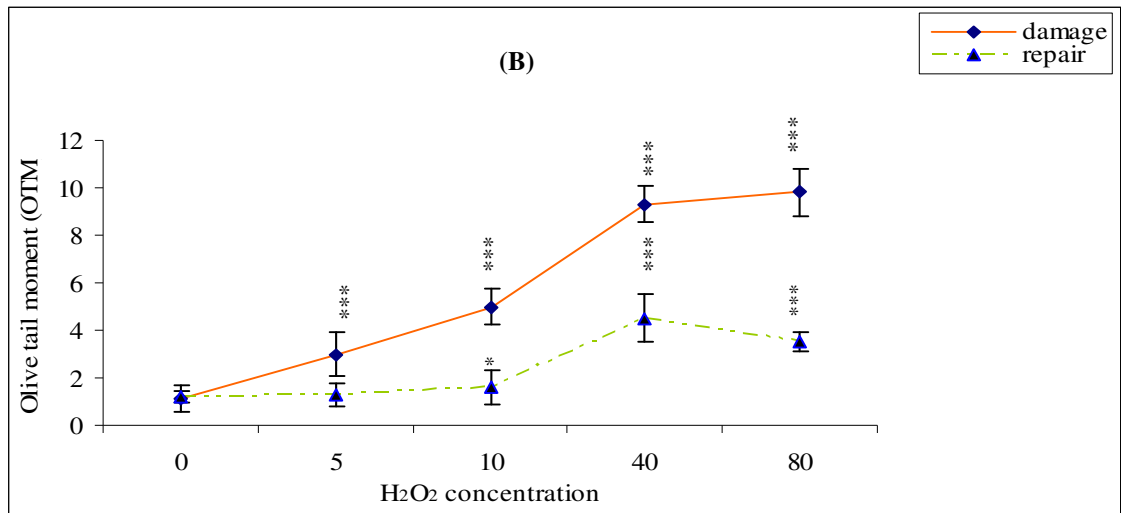
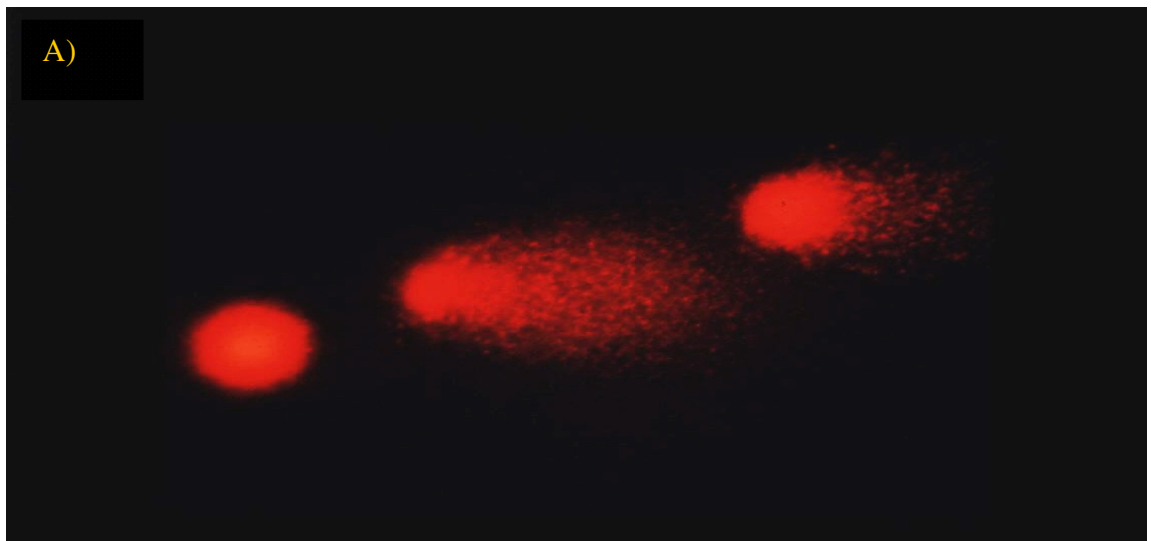


Figure 3.2B The dose response of DNA damage and repair in lymphocytes obtained from healthy donors and treated with different H₂O₂ concentrations. Results of 3 repeats at each H₂O₂ concentration for damage were compared with those for repair by one way ANOVA in SPSS 15 (* $p < 0.05$, and *** $p < 0.001$)



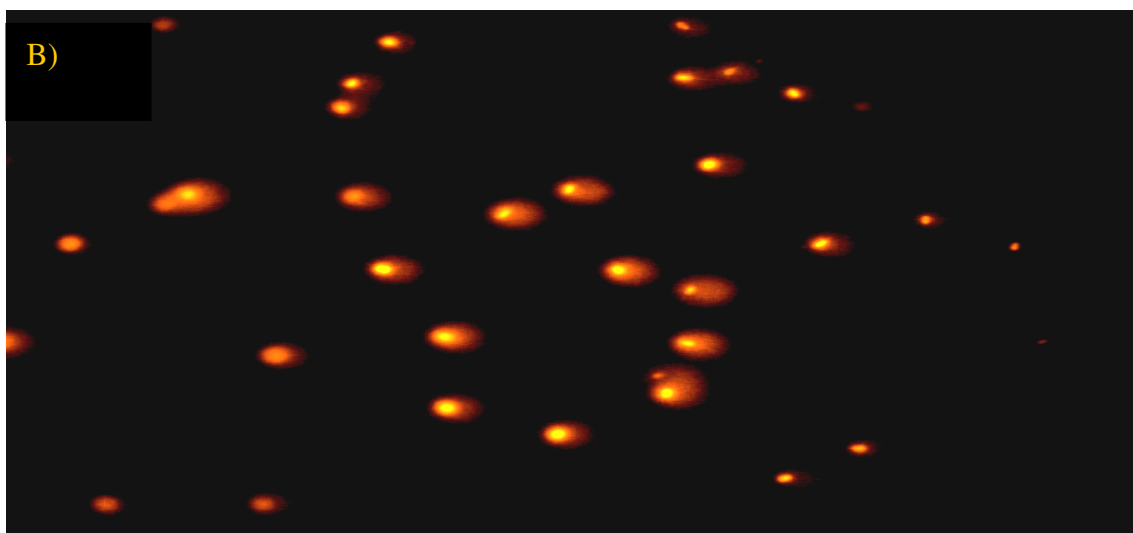


Figure 3.3 DNA damage in A) lymphocytes and B) sperm. Viewing magnification x200

3.3.2 Effect of nanoparticles on human lymphocytes and sperm treated under different illumination conditions

3.3.2.1 Particles characterization with SEM

Zinc oxide (ZnO) and TiO₂ were characterized with SEM as described in chapter 2 section 2.2.1.1 and found to be ZnO 40 and TiO₂ 70 nm.

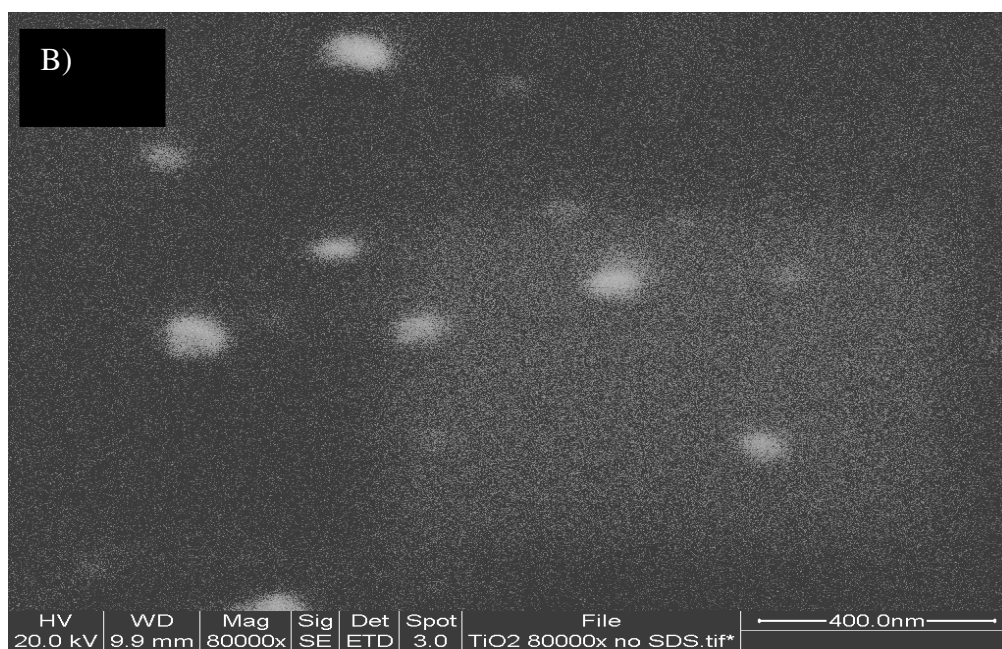
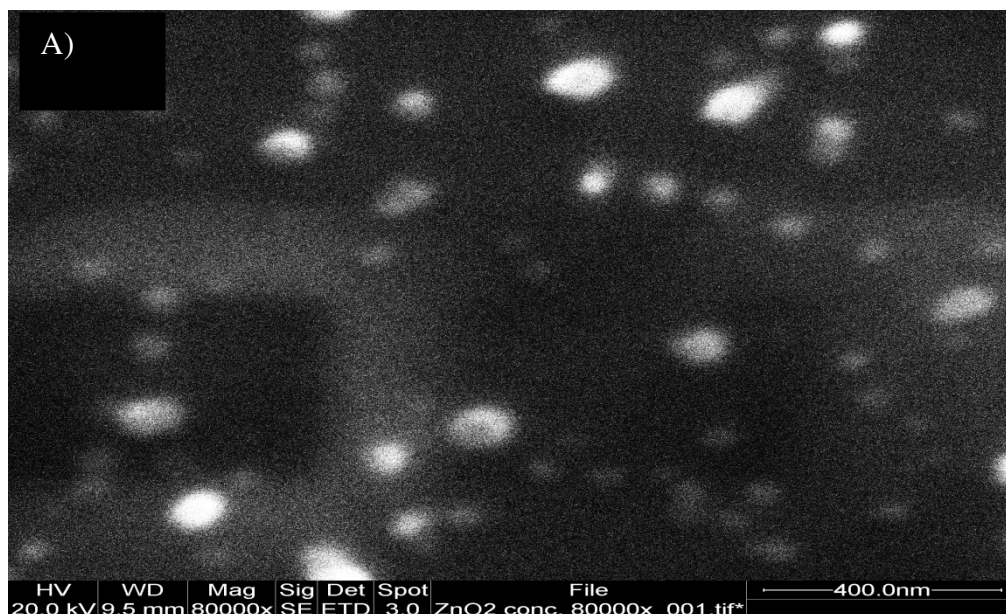


Figure 3.4: Scanning electron micrograph of A) ZnO NPs (40 nm) and B) TiO₂ NPs (70 nm)

3.3.2.2 ZnO nanoparticles

In general, the sperm percentage head DNA showed a progressive reduction with increasing concentrations of ZnO nanoparticles after treatment in the dark, and for PI as well as SI samples. However, the percentage reduction was greater for PI and SI samples compared with samples treated in the dark (table 3.1 and figure 3.5A).

For lymphocytes, the SI and PI samples showed a higher level of damage compared with the samples treated in the dark (table 3.1 and figure 3.5B). In both cell types, there was damage that was not as great as that in the positive control. By comparison with the negative control, all values were highly statistically significant for sperm except at the lowest concentration in the dark, which was not significant, and for SI samples at the lowest concentration, which was significant only at the 5% level (table 3.1 and figures 3.5 A & B).

With regard to photo-genotoxicity, responses for sperm treated in PI and SI incubation conditions were not statistically significant compared to responses of sperm treated in the dark, except at the lowest concentration for SI samples. However, for lymphocytes, responses were statistically significant at the highest concentration for both PI and SI samples (table 3.1 and figures 3.5 A & B).

Table 3.I: Effect of ZnO nanoparticles on % head DNA of sperm and Olive tail moments for lymphocytes

ZnO concentration (µg/ml)	Nc		11.5		46.2		69.4		92.3		Pc	
	Median	Mean	Median	Mean	Median	Mean	Median	Mean	Median	Mean	Median	Mean
Sp D	76.38	75.99 ±1.15	76.71ns	74.25 ±2.20	71.69***	70.53 ±1.62	70.98***	68.41 ±1.33	66.2***	66.33 ±1.59	57.48***	58.78 ±1.46
Sp PI	76.39	75.45 ±1.26	72.71***	71.54 ±1.37	71.28***	72.81 ±1.49	67.07***	64.65 ±1.59	63.45***	63.52 ±1.72	59.59***	63.8 ±1.82
Sp SI	78.23	78.79 ±1.75	69.46*** (***)	69.97 ±1.68	68.75ns	68.68 ±1.48	66.03ns	67.73 ±1.81	64.51**	63.41 ±1.88	57.81***	59.55 ±1.88
Ly D	2.14	2.65 ±0.30	2.87***	4.17 ±0.53	3.54***	4.66 ±0.60	5.33***	6.25 ±0.59	5.24***	6.46 ±0.71	9.76***	9.81 ±0.71
Ly PI	2.03	2.89 ±0.42	3.59***	5.04 ±0.63	4.37***	6.33 ±0.79	4.39***	5.62 ±0.66	6.20***	8.25 ±0.71	10.45*** (***)	11.04 ±0.74
Ly SI	1.95	2.99 ±0.44	2.02*	3.79 ±0.62	3.99***	5.85 ±0.72	5.22***	6.51 ±0.55	6.25***	8.36 ±0.91	7.29*** (***)	9.74 ±1.17

Nc= negative control of untreated cells

Pc= positive control of H₂O₂ (80 µM/ 2.72 µg/ml)

Sp D = sperm: Treatment in dark

Sp PI = sperm: Treatment after pre-irradiation with UVA

Sp SI = sperm: Treatment with simultaneous UVA irradiation

Ly D = Lymphocytes: Treatment in dark

Ly PI = Lymphocytes: Treatment after pre-irradiation with UV

Ly SI = Lymphocytes: Treatment with simultaneous UV irradiation

When comparing D, PI and SI for different ZnO concentrations with negative control: ns = not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. In parentheses, when comparing PI and SI with D. (***) $p < 0.001$.

Figure 3.5 Effect of UVA irradiation on human sperm and lymphocytes treated with ZnO nanoparticles

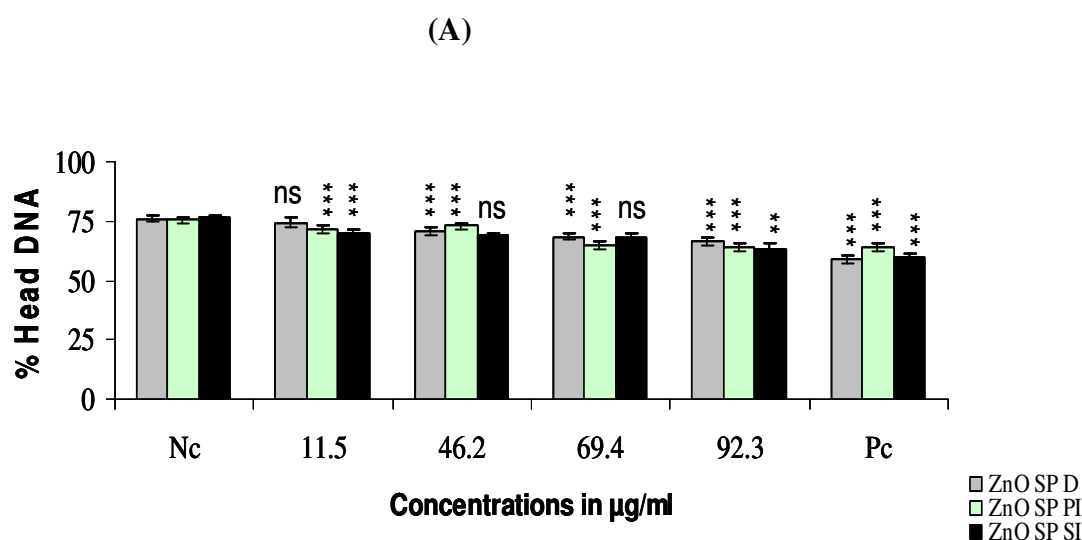


Figure 3.5A Effect of ZnO nanoparticles as well as negative control of untreated sperm cells (Nc) and positive control (Pc) of 80 μ M (2.72 μ g/ml) H_2O_2 on human sperm treated in the dark (D), after pre-irradiation with UV (PI) and with simultaneous irradiation with UV (SI). Significance values are shown in Table 3.1. The values represent the means of three separate experiments combined. Bars indicate standard errors.

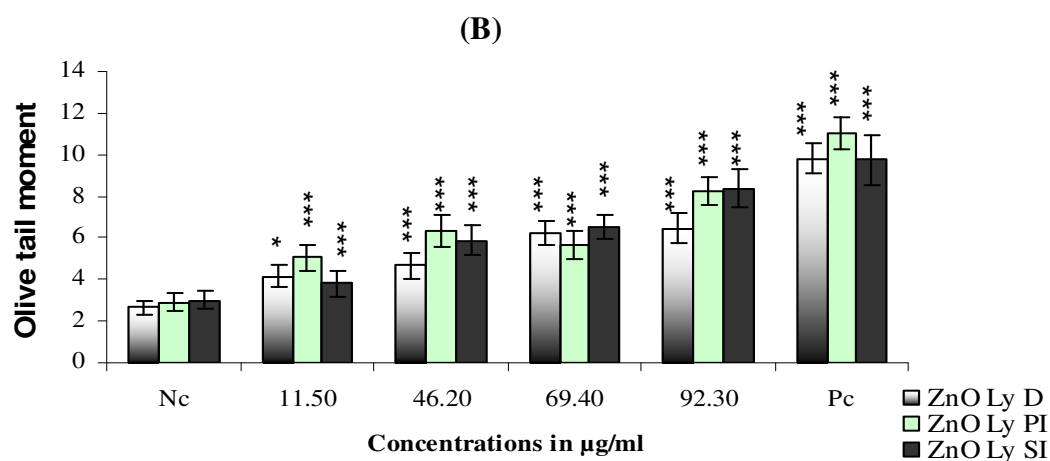


Figure 3.5B Effect of ZnO nanoparticles as well as negative control of untreated lymphocyte cells (Nc) and positive control (Pc) of 80 μ M (2.72 μ g/ml) H_2O_2 on human lymphocytes treated in the dark (D), after pre- irradiation with UV (PI) and with simultaneous irradiation with UV (SI). Significance values are shown in Table 3.1. The values represent the means of three separate experiments combined. Bars indicate standard errors.

3.3.2.3 TiO₂ nanoparticles

With TiO₂, the % head DNA showed damage which tended to plateau at the highest concentration of 59.7µg/ml for sperm for all treatments (figure 3.6A and table 3.2).

However, in lymphocytes there was no tendency towards plateau formation with the highest concentration. The maximum damage was observed in SI samples throughout the concentration range followed by the PI samples. Those samples treated in the dark had lower values for Olive tail moment (figure 3.6B and table 3.2). For both sperm and lymphocytes, all values by comparison with the negative control were highly significant (figure 3.6A&B and table 3.2).

With regard to photo-genotoxicity, there were no statistically significant responses for the sperm when the results for PI and SI samples were compared to responses in the dark, except at the lowest dose for PI samples. However, for lymphocytes, responses were statistically significant at the highest concentration after treatment for both PI and SI samples. For SI samples there was a concentration – related increase over the whole dose range and the highest three doses were statistically significantly different by comparison with treatment in the dark (figure 3.6A &B and table 3.2).

Table 3.2: Effect of TiO₂ nanoparticles on percentage head DNA of sperm and Olive tail moments for lymphocytes

TiO ₂ concentration µg/ml	Nc		3.73		14.92		29.85		59.7		Pc	
	Median	Mean	Median	Mean	Median	Mean	Median	Mean	Median	Mean	Median	Mean
Sp D	87.49	85.98 ±1.32	77.59***	75.55 ±1.82	69.66***	71.64 ±1.35	65.00***	66.83 ±1.40	67.92***	68.57 ±1.32	61.70***	60.03 ±2.50
Sp PI	75.39	75.69 ±1.55	59.83*** (***)	61.50 ±2.54	68.29***	71.29 ±2.31	68.99**	67.88 ±1.66	67.36***	68.98 ±1.94	61.43***	62.58 ±1.73
Sp SI	78.85	78.79 ±1.75	74.12***	73.90 ±1.75	71.39***	72.19 ±1.38	69.23***	67.59 ±2.31	68.45***	69.46 ±1.32	57.95***	59.62 ±2.51
Ly D	0.84	1.9 ±0.29	2.45***	3.17 ±0.31	2.5***	3.43 ±0.43	2.85***	3.67 ±0.44	6.05***	6.16 ±0.51	5.62***	6.99 ±0.65
Ly PI	1.24	2.52 ±0.4	2.44***	3.08 ±0.39	3.5***	4.4 0±0.52	6.49***	6.44 ±0.48	6.17*** (***)	7.30 ±0.81	6.60***	8.81 ±0.75
Ly SI	1.83	2.98 ±0.35	2.74**	4.31 ±0.35	4.74*** (***)	7.33 ±0.47	6.22*** (***)	8.97 ±0.46	10.44*** (***)	10.66 ±0.66	9.95***	10.88 ±0.70

Nc= negative control of untreated cells

Pc= positive control of H₂O₂ (80 µM/ 2.72 µg/ml)

Sp D = sperm: Treatment in dark

Sp PI = sperm: Treatment after pre-irradiation with UVA

Sp SI = sperm: Treatment with simultaneous UVA irradiation

Ly D = Lymphocytes: Treatment in dark

Ly PI = Lymphocytes: Treatment after pre-irradiation with UV

Ly SI = Lymphocytes: Treatment with simultaneous UV irradiation

When comparing D, PI and SI with negative control: ** $p < 0.01$; *** $p < 0.001$. In parentheses, when comparing PI and SI with D. (***) $p < 0.001$

Figure 3.6 Effect of UVA irradiation on human sperm and lymphocytes treated with TiO₂ nanoparticles

(A)

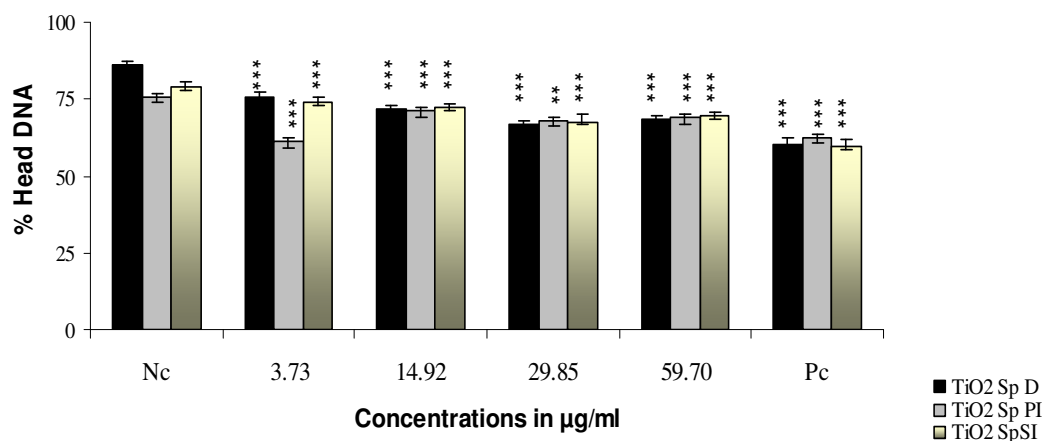


Figure 3.6A Effect of TiO₂ nanoparticles as well as negative control of untreated sperm cells (Nc) and positive control (Pc) of 80 µM (2.72 µg/ml) H₂O₂ on human sperm treated in the dark (D), after pre-irradiation with UV (PI) and with simultaneous irradiation with UV (SI). Significance values are shown in Table 3.2. The values represent the means of three separate experiments combined. Bars indicate standard errors.

(B)

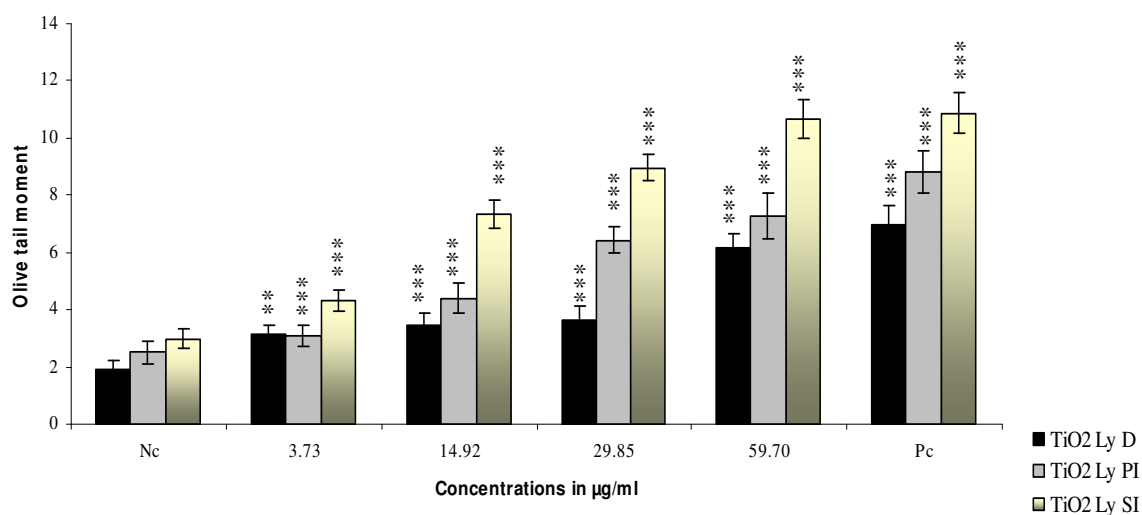


Figure 3.6B Effect of TiO₂ nanoparticles as well as negative control of untreated lymphocyte cells (Nc) and positive control (Pc) of 80 µM (2.72 µg/ml) H₂O₂ on human lymphocytes treated in the dark (D), after pre- irradiation with UV (PI) and with simultaneous irradiation with UV (SI). Significance values are shown in Table 3.2.

The values represent the means of three separate experiments combined. Bars indicate standard errors.

3.4 Discussion

Nanotechnology has numerous useful applications in a variety of fields which has attracted publicity, however, many of these nanoparticles are non-biodegradable and their long-term behaviour is not well understood (Braydich-Stolle et al., 2005).

The aquatic toxicity screening studies in a 72h acute test involving the green algae *Pseudokirchneriella subcapitata* have concluded that ultra-fine TiO₂ (median particle size 140 nm) has a low hazard potential (Warheit et al., 2007a). However, investigations on toxicity of TiO₂, SiO₂ and ZnO water suspensions towards bacteria (*Bacillus subtilis*, *Escherichia coli*) and the eukaryotic *Daphnia magna* have revealed that these were hazardous to all test organisms, with toxicity increasing with particle concentration. Toxicity of the three compounds decreased from ZnO to TiO₂ to SiO₂ (Adams et al., 2006a; Adams et al., 2006b). Zhu et al. (2008) have studied developmental toxicity of metal oxide nanoparticles, and the results have demonstrated that nanoparticles of ZnO were very toxic to zebrafish embryos and larvae.

Turkez and Geyikoglu (2007) have reported that TiO₂ increased sister chromatid exchanges (SCE) and micronuclei (MN) frequencies in human whole blood cultures suggesting that TiO₂ can enhance oxidative stress mediated DNA damage. As particle size decreases, surface area increases leading to inflammatory response for, e.g., in lung tissue (Brown et al. 2001). Studies investigating the genotoxic capacity of coal fly ash have shown a role for particulate size and iron release leading to radical generation and oxidative DNA damage in human lymphocytes and human airways epithelial cells (Kleijnans et al., 1989; Prahalad et al., 1999).

The bactericidal action of photo-activated TiO₂ particles have also been demonstrated on contaminated dental implants (Suketa et al. 2005) and metal pins used for skeletal traction (Tsuang et al. 2008). Studies involving intravenous or intra abdominal administration of nanoparticles have reported their accumulation in brain and testes indicating their ability in crossing the blood-brain and blood-testis barriers (Chen et al. 2003; Borm and Kreyling 2004).

Some nanoparticles have their activity enhanced by UV and other catalysts used as endogenous antioxidants (Nakagawa et al., 1997). Hence, their potential to cause undesirable problems for man and the environment by virtue of their size and properties should not be under-estimated (Hoet et al., 2004). For soluble chemicals, as early as 1978, dichlorobromopropane resulted in lower sperm counts because of the destruction of undifferentiated spermatogonia (Potashnik et al., 1978).

A decrease in human semen quality was reported in 1992 and oestrogen-like compounds were suspect (Carlsen et al., 1992). With increasing use of nanoparticles, one area of concern could be the effect of the nanoparticles on germ cells, as these could have serious implications on the health of future generations.

The study with H₂O₂ on lymphocytes and sperm in the Comet assay showed that the system was capable of detecting DNA damage and repair induced by chemicals activity through oxygen radical generation mechanisms. Thus it was considered suitable for use with ZnO and TiO₂ nanoparticles which might also induce oxygen radical mechanisms. The present investigation suggests that both ZnO and TiO₂ are capable of inducing genotoxic effects on human sperm and lymphocytes and that the effect of ZnO is enhanced by UVA both in case of lymphocytes and sperm, but effects are only

statistically significantly different from responses in the dark at the highest doses after pre-irradiation and simultaneous irradiation. However, TiO₂ tended not to produce any photogenotoxic effects in sperm, but TiO₂ does have some photogenotoxic effects with lymphocytes, especially after simultaneous irradiation with UVA, where responses were dose-related. Photo-genotoxic effects have been produced in the absence of overt toxicity in contrast to the findings of Nakagawa et al. (1997), and Kamat et al. (1998), where toxicity was reported. The mixed results of TiO₂ nanoparticle toxicity have been attributed to the unique clustering property of TiO₂ nanoparticles under different conditions of pH and ionic strength (Baveye and Laba, 2008).

The probable cause could be the oxygen radicals generated from these oxides, which could act synergistically with UVA to exacerbate the responses. For ZnO the mechanism could be the dissolution of Zn²⁺ (Brunner et al., 2006; Schrand et al., 2010). Although the exact mechanism is unknown, studies with vitamin C could confirm the mechanism of free radical generation.

Chapter 4

Genotoxicity and cytotoxicity of zinc oxide and titanium dioxide in HEp-2 cells

4.1 Introduction

Nanoparticles have become very attractive for commercial and medical products in the last decade because of their various properties. Nanoparticles have been widely used for manufacturing many consumer products such as electronics, computers, food colorants or clothes, arriving on the market at a rate of 3-4 per day (PEN, 2008). In medical fields such as oncology, cardiovascular medicine, molecular diagnostics, drug discovery and drug delivery nanoparticles are also increasingly used (Kubik et al., 2005; LaVan et al., 2003; Leary et al., 2006). One of the future intended usages of titanium dioxide (TiO_2) is for artificial orthopaedic implants (Schuler et al., 2006). Most recently, titanium dioxide nanoparticles chemically linked to antibodies via bivalent dihydroxybenzene, so-called phototoxic "nanobio hybrids", were used to selectively kill glioblastoma multiform brain cancer cells (Rozhkova et al., 2009). By conventional definition one dimension of nanoparticles has to be < 100 nm, however, due to their biological uniqueness not being constrained by this definition the pharmaceutical industry uses sizes of 1-1000 nm to describe nanomaterials (Sun et al., 2004; Teeguarden et al., 2007). Nanoparticle properties are rather different from bulk materials and these differences include their small size in the nanometre range, a large surface area to volume ratio (Oberdorster et al., 2005) and unique physico-chemical properties. Consequently, nanoparticles can easily pass through cell membranes and may show unpredictable toxic effects. The major toxicological concern arising from this is that many nanoparticles which cross cell membranes become lodged in mitochondria (Foley et al., 2002) with some of the engineered nanomaterials also being redox active (Colvin, 2003). Hence, the increasing applications of nanoparticles in various fields may lead to an increase in human exposure which in turn may increase direct toxicological effects. Despite apparent advantages of certain nanoparticles and their applications, many

undesirable side effects of nano-sized particles have been documented in previous studies over the last decade (Goldston, 2007; Igarashi, 2008).

Nanoparticles such as zinc oxide (ZnO) and TiO₂ are increasingly used in a variety of industrial and medical applications including high-tech materials, plastics, paints and production of paper, and also very frequently in cosmetics and sun screens (Wang et al., 2007a). Zinc oxide and TiO₂ nanoparticles with sizes between 50–500 nm are employed to increase the sun protection factor (SPF) of e.g. sunscreens due to their scattering properties, which enables them to act as nano-mirrors reflecting UV light and as UV filters against sun radiation. In addition to UV reflection properties, their small size is also used as an advantage to improve homogeneity of distribution in cosmetics. However, TiO₂, in spite of its reflection and scattering properties to UVB and UVA, also absorbs about 70% of the total irradiated UV light (Dunford et al., 1997).

The cytotoxic effects of TiO₂ nanoparticles on human A549 lung epithelial cells were accompanied by the active up-take of TiO₂ nanoparticles by endocytosis (Stearns et al., 2001). In animals, *in vivo* studies have also shown that exposure and inhalation of TiO₂ particles can induce cytotoxicity in lung cells with subsequent inflammatory responses in lung tissue being directly related to the particle size (Dick et al., 2003; Monteiller et al., 2007; Rehn et al., 2003; Renwick et al., 2004; Warheit et al., 2007b). Garabrant and colleagues noted that 17% of the workers exposed to TiO₂ were affected by pleural diseases, which were proportionate to the duration of work in TiO₂ manufacturing (Garabrant et al., 1987). *In vitro* studies on human sperm and lymphocytes also showed that both nano-sized ZnO and TiO₂ (10-100 µg/ml) were genotoxic to both cell types within the Comet assay in the absence of toxicity (Gopalan et al., 2009), the same was true for bacteria and vertebrates (Brayner et al., 2006; Heinlaan et al., 2008). In

addition, *in vitro* studies of ZnO in Chinese hamster ovary (CHO) cells and in human epidermal cell lines have revealed clastogenic properties (Dufour et al., 2006; Sharma et al., 2009). Studies on lymphocytes treated with TiO₂ or ZnO nanoparticles showed significantly increased DNA breakage and micronucleus formation (Gopalan et al., 2009; Kang et al., 2008). Effects of TiO₂ nanoparticles on non-human mammalian cells were also reported, e.g. exposure of rat liver cells to TiO₂ nanoparticles resulted in cytotoxicity (Hussain et al., 2005). Titanium dioxide also induced apoptosis and micronuclei in Syrian hamster embryo fibroblasts (Rahman et al., 2002). Various nanoparticles have shown some cytotoxicity and/or genotoxicity (Dhawan et al., 2006; Lewinski et al., 2008; Singh et al., 2007; Wang et al., 2007a, b), and different mechanisms of damage induction have been suggested including radical oxygen species (ROS) and lipid peroxidation, as well as alteration of signal transduction (Shrivastava et al., 2007), but the exact mechanism(s) has (have) yet to be found.

Signal transduction pathways are important mechanisms through which the cell coordinates particular functions. Trans-membrane receptors receive and then deliver signals from the extracellular lumen via different mechanisms into the nucleus (Wang et al., 2003). Tyrosine phosphorylation is the major signalling pathway through which receptor tyrosine kinases catalyze the transfer of a phosphate from ATP to the hydroxyl group on tyrosine residues of protein substrates in turn mediating cell growth, differentiation, host defence, and metabolic regulations (Hubbard et al., 1998). The importance of tyrosine phosphorylation events in the presence of toxic nanoparticles was previously reported in a study characterizing the anti-bacterial effects of novel silver nanoparticles, which were able to penetrate the bacteria and subsequently modulate the phosphotyrosine profile (Shrivastava et al., 2007).

In this study, the cytotoxicity and genotoxicity of ZnO and TiO₂ nanoparticles was investigated using the human epithelial HEp-2 cell line. In addition the tyrosine phosphorylation events caused by ZnO and TiO₂ occurring simultaneously to cytotoxicity and or genotoxicity was also investigated.

4.2 Materials and Methods

4.2.1 Cell culture of the HEp-2 cell line

HEp-2 cells (HeLa derivative) were cultured in EMEM-EBBS medium. For details refer to section 2.2.2.1.

4.2.2 Chemicals

All chemicals used have been mentioned in chapter 2 section 2.2.2.2

4.2.3 Particle preparation and characterization

Zinc oxide and titanium dioxide nanoparticles were suspended in 10 ml EMEM-EBBS medium at concentrations of 10, 20, 50, and 100 µg/ml. Suspensions were probe-sonicated. The resulting ZnO and TiO₂ suspensions were measured as a function of incubation time. Prior to evaluation of the toxic potential, the mean size (hydrodynamic diameter) of the nanoparticles was determined using a high performance particle sizer (Malvern Instruments Ltd., Worcestershire, U.K.) (details in section 2.2.2.3).

4.2.4 Cytotoxicity assays

4.2.4.1 Mitochondrial activity assay

The MTT assay to evaluate the mitochondrial activity was done according to the originally described method (Mosmann, 1983) (details in section 2.2.2.4.1).

4.2.4.2 Neutral red uptake (NRU) assay

The NRU assay was done to determine the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells (Borenfreund and Puerner, 1985) details in section 2.2.2.4.2.

4.2.5 Cell viability

Prior to the Comet assay, cells were incubated with ZnO or TiO₂ nanoparticles for 4 h at different concentrations (10, 20, 50, 100 µg/ml) and assayed for viability using the Trypan blue dye exclusion test. The cut-off point was 75% as suggested by Henderson and colleagues (Henderson et al., 1998) details in section 2.2.2.5.1.

4.2.6 Genotoxicity assays

4.2.6.1 Comet assay

The sub-confluent monolayer was exposed to four different concentrations of ZnO and TiO₂, and 80 µM/ml of hydrogen peroxide (H₂O₂) as a positive control (Pc). There was also a negative control using untreated cells (Nc). After an incubation of 4 hours for NPS and 30 minutes for H₂O₂, cells were washed with cold PBS and harvested with trypsin-EDTA and followed by a centrifugation. The pellet was finally resuspended in PBS. The Comet slides were prepared by the method as previously described (Tice et al., 2000) (details in section 2.2.2.5).

4.2.6.2 *In vitro* cytokinesis-block micronucleus (CBMN) assay

The CBMN assay was performed as described previously (Fenech, 1993). HEp-2 cells were grown in 6-well cell culture plates suspended in 2 ml EMEM medium at a concentration of 1×10^5 . After 24 hours of culture, either the TiO₂ nanoparticles (10, 20 and 50 µg/ml) or ZnO nanoparticles (10, 20, 50 and 100 µg/ml) or mitomycin C (positive control; 0.4 µM = 0.134 µg/ml) were added to the cultures. An untreated culture served as the negative control for details refer to section 2.2.2.6.

4.2.7 Detection of tyrosine phosphorylation in HEp-2 cells

A sub-confluent HEp-2 monolayer was treated with different concentrations (0, 10, 20, 50 and 100 µg/ml) of ZnO or TiO₂ nanoparticles and incubated for 4 hours. Untreated cells served as a negative control. Viability and membrane integrity was determined using the Trypan blue dye exclusion test. Protein was quantified using the Bio-Rad assay (Lowry et al, 1951). The cell lysate was suspended in Laemli sample buffer and equal proteins (35 µg) protein from each sample was loaded on SDS gel and detected by the immunoblotting technique using a phosphotyrosine mouse monoclonal primary antibody (P-Tyr-100) and an anti-mouse IgG-HRP linked secondary antibody details in section 2.2.2.7.

4.2.8 Statistical analysis

The means from MTT and NRU data were compared to the means of the negative control data using the one-way ANOVA test. A Mann-Whitney U-test was used for the Comet assay. For the CBMN assay, the Chi square test and Fisher exact test were used to compare the number of MN in the treated samples to the negative control of untreated

HEp-2 samples. The threshold of significance was $p < 0.05$. Values at or below this were considered significant.

4.3 Results

4.3.1 Particle characterization

Dynamic light scattering (DLS) measurements of ZnO and TiO₂ nanoparticle suspensions (figure 4.1), were obtained with a high performance particle sizer. Using DLS, particle sizes were observed to be widely distributed. The increase in size (aggregation) occurred with increasing concentrations but remained constant over a 48-hour period except at higher concentrations of TiO₂ (Table 4.1). Although the particle size considerably increased as a function of concentration it was minimally increased as a function of time.

Table 4.1: Physical characterization of ZnO and TiO₂ nanoparticles using a Zeta sizer. Shown are the sizes of nanoparticles in nanometers measured for different ZnO and TiO₂ concentrations in cell culture media for different incubation times. The values generated by the Zeta sizer originate from an average of three measurements each with three runs.

Incubation periods in hours		Particle size [nm] at concentrations of			
		10 µg/ml	20 µg/ml	50 µg/ml	100 µg/ml
ZnO	0 h	17.0	307.1	419.9	406.2
	2 h	17.9	151.6	369.9	399.6
	4 h	24.7	281.3	373.0	389.2
	2 h	19.0	257.4	319.6	355.7
	48 h	30.8	308.6	370.0	397.9
TiO ₂	0 h	143.0	383.6	440.7	507.5
	2 h	313.9	399.1	403.2	577.5
	4 h	339.2	426.4	475.7	567.9
	2 h	400.0	403.2	399.1	662.7
	48 h	383.6	383.6	507.5	721.8

A)

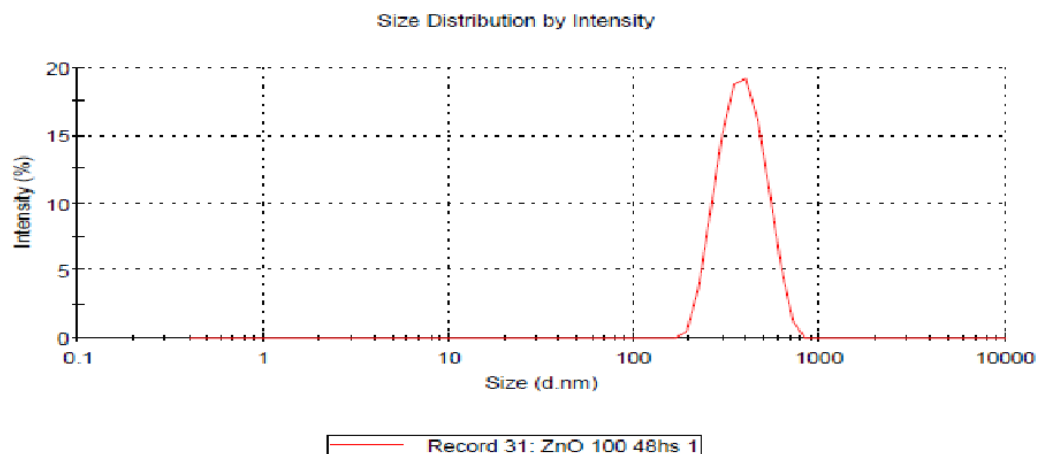


Figure 4.1A The mean hydrodynamic diameter of ZnO NPs suspended in EMEM-EBBS at a concentration of 100 $\mu\text{g/ml}$ for 48 hours. The mean hydrodynamic diameter as determined by DLS (dynamic light scattering) is 397.9 nm.

B)

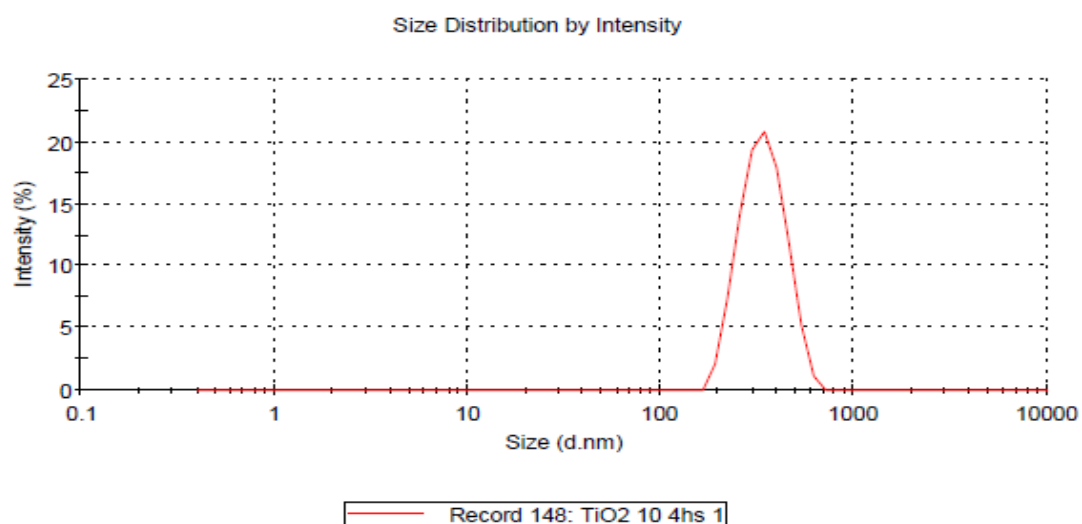


Figure 4.1B The mean hydrodynamic diameter of TiO_2 NPs suspended in EMEM-EBBS at a concentration of 10 $\mu\text{g/ml}$ for 4 hours. The mean hydrodynamic diameter as determined by DLS (dynamic light scattering) is 339.2 nm.

4.3.2 Cytotoxicity

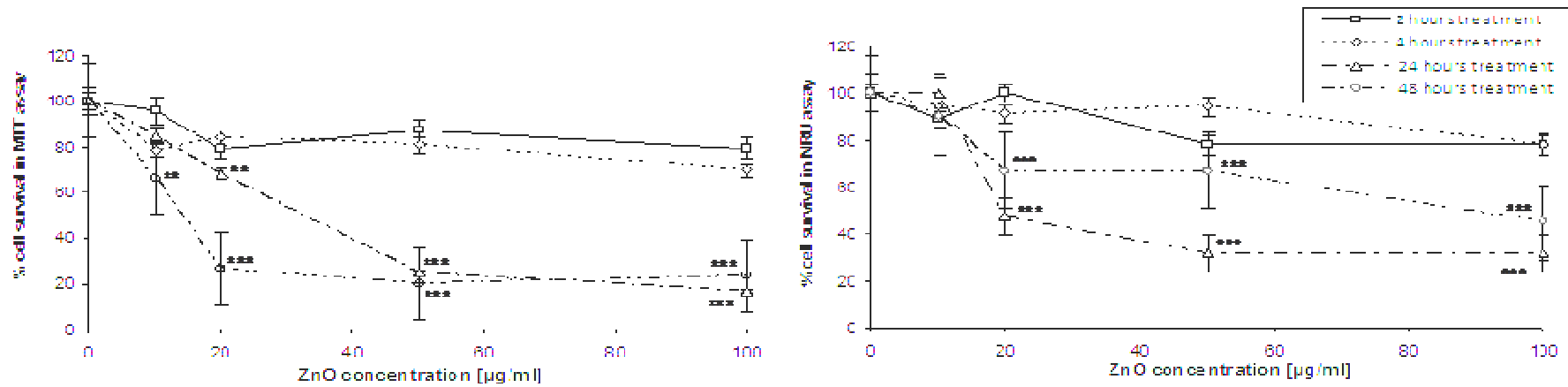
4.3.2.1 The MTT assay and the NRU assay

The results demonstrated a concentration- and time-dependent cytotoxicity as measured by optical density (OD) at 570 nm after exposure to ZnO or TiO₂ nanoparticles which was evident for ZnO but not for TiO₂ due to precipitation after 24 h (Figure 4.2). The percentage (%) cell survival in the MTT assay compared to the untreated control was decreased dramatically in 24 and 48 hours in ZnO treated cells reaching ~16% at the highest ZnO concentration (100 µg/ml). However, the highest concentrations of TiO₂ (50 and 100 µg/ml) were precipitated within 24 hours as the results for the OD showed higher readings compared with the negative control (> 140%).

The NRU assay showed similar results as the MTT assay and demonstrated a concentration and time dependent cytotoxicity after exposure to ZnO or TiO₂ nanoparticles for more than 4 hours (Figure 4.2 and tables 4.2 & 4.3).

Optical density (OD) means \pm SD of triplicates MTT assays on HEp-2 cells treated with different ZnO and TiO₂ concentrations and for different times as well as untreated controls.

A)



B)

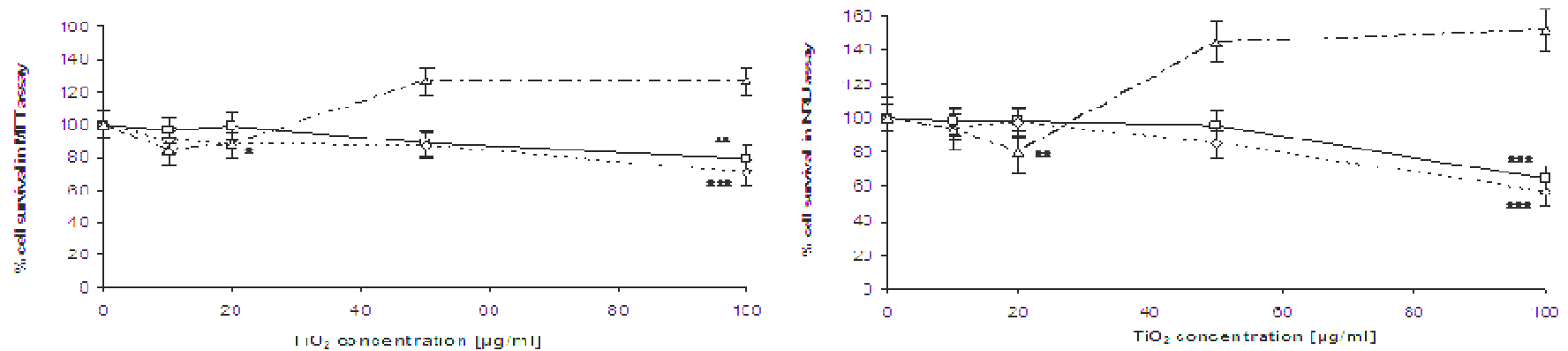


Figure 4.2 Cytotoxicity of ZnO A) and TiO₂ B) nanoparticles on human HEP-2 cells treated for different times for ZnO (2, 4, 24, and 48 hours) and for TiO₂ (2, 4, and 24 hours), was measured using the MTT and the NRU assays. The % cell survival compared to the negative control of untreated HEP-2 cells was evaluated for different concentrations of ZnO and TiO₂ nanoparticles. The values represent the mean of three separate experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Bars indicate standard errors

Table 4.2: Optical densities from MTT assays of HEp-2 cells treated with different concentrations of ZnO and TiO₂. Optical density (OD) means \pm SD of triplicates MTT assays on HEp-2 cells treated with different ZnO and TiO₂ concentrations and for different times as well as untreated controls (Nc). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Treatment in hours		Concentration in $\mu\text{g/ml}$				
		Nc	10	20	50	100
ZnO	2	1.16 \pm 0.37	1.11 \pm 0.04	0.92 \pm 0.26	1.01 \pm 0.28	0.92 \pm 0.32
	4	1.26 \pm 0.36	0.98 \pm 0.49	1.05 \pm 0.23	1.01 \pm 0.47	0.92 \pm 0.56
	24	0.97 \pm 0.26	0.82 \pm 0.16	0.66 \pm 0.26 **	0.24 \pm 0.21 ***	0.16 \pm 0.04 ***
	48	0.76 \pm 0.46	0.37 \pm 0.14 **	0.15 \pm 0.06 ***	0.11 \pm 0.05 ***	0.13 \pm 0.02 ***
TiO ₂	2	0.81 \pm 0.19	0.78 \pm 0.14	0.80 \pm 0.28	0.72 \pm 0.16	0.63 \pm 0.13 **
	4	0.91 \pm 0.24	0.82 \pm 0.24	0.80 \pm 0.14	0.79 \pm 0.11	0.64 \pm 0.09 ***
	24	0.64 \pm 0.09	0.59 \pm 0.08	0.53 \pm 0.08 *	0.81 \pm 0.04	0.82 \pm 0.11

Table 4.3: Optical densities from NRU assays of HEp-2 cells treated with different concentrations of ZnO and TiO₂. Optical density (OD) \pm SD means of triplicates NRU assays on HEp-2 cells treated with different ZnO and TiO₂ concentrations and different time as well as untreated controls (Nc). ** $p < 0.01$, *** $p < 0.001$

Treatment in hours		Concentration in $\mu\text{g/ml}$				
		Nc	10	20	50	100
ZnO	2	0.18 \pm 0.08	0.17 \pm 0.06	0.19 \pm 0.08	0.15 \pm 0.04	0.15 \pm 0.03
	4	0.70 \pm 0.07	0.66 \pm 0.08	0.64 \pm 0.10	0.66 \pm 0.15	0.62 \pm 0.06
	24	0.20 \pm 0.11	0.20 \pm 0.09	0.09 \pm 0.04 ***	0.07 \pm 0.01 ***	0.07 \pm 0.01 ***
	48	0.11 \pm 0.02	0.10 \pm 0.02	0.07 \pm 0.01 ***	0.07 \pm 0.01 ***	0.08 \pm 0.02 ***
TiO ₂	2	0.50 \pm 0.09	0.49 \pm 0.08	0.49 \pm 0.08	0.48 \pm 0.04	0.32 \pm 0.11 ***
	4	0.72 \pm 0.11	0.68 \pm 0.09	0.68 \pm 0.11	0.65 \pm 0.09	0.41 \pm 0.13 ***
	24	0.45 \pm 0.08	0.42 \pm 0.06	0.36 \pm 0.07 **	0.65 \pm 0.09	0.68 \pm 0.11

4.3.3 Cell viability and membrane integrity

The cell viability in the Comet assay and immunoblotting assay using the Trypan blue dye exclusion assay was between 70-85% (mostly ~ 85%) for all ZnO and TiO₂ samples, with the exception of the highest dose of TiO₂ (100 µg/ml), which showed 65% viability. All doses examined, therefore had high membrane integrity.

4.3.4 Genotoxicity

4.3.4.1 DNA damage

The parameters of Olive tail moment (OTM) and % tail DNA indicated significant ($p < 0.05$) dose-related DNA damage in HEp-2 cells. Exposure for 4 h to different concentrations of ZnO or TiO₂ nanoparticles showed a 3-fold increase for ZnO and a 2-fold increase for TiO₂ in DNA damage compared to the negative control of untreated HEp-2 cells. However, for the lowest ZnO dose the induced damage, measured in % tail DNA, did not reach significance levels, but did for the OTM (refer to table 4.4 and figure 4.3).

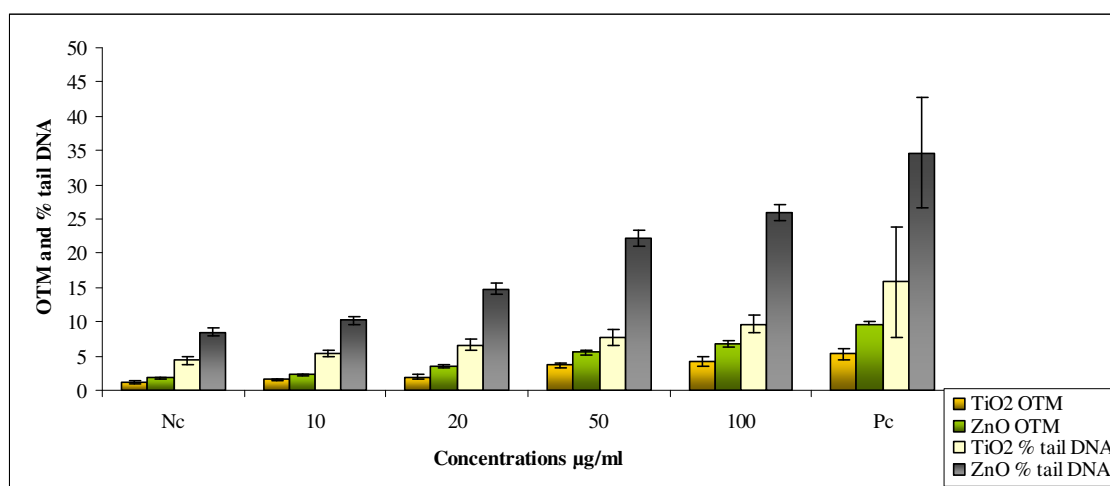


Figure 4.3: The effect of TiO₂ or ZnO nanoparticle exposure on DNA damage in HEP-2 cells as measured by the Comet assay parameters, mean Olive tail moment and % tail DNA. Cells were exposed to TiO₂ or ZnO at concentrations of 10, 20, 50, or 100 µg/ml or a negative control of untreated Hep-2 cells (Nc) for 4 hours. The positive control (Pc) was treated with 80 µM (2.72 µg/ml) H₂O₂ for 30 minutes. Significance values are shown in Table 4.4. The values represent the means of three separate experiments. Bars indicate standard errors.

Table 4.4: Effect of ZnO and TiO₂ nanoparticles on HEP-2 cells in the Comet assay. The DNA damage was evaluated using the Comet assay parameter, mean Olive tail moment and % tail DNA. The values resulted from three independent experiments. Nc= negative control of untreated Hep-2 cells, Pc= positive control of H₂O₂ 80 µM, ns = non significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

Treatment	concentration µg/ml	Comet parameter DNA damage	
		OTM	% tail DNA
Zno	Nc	1.76 ± 0.26	8.51 ± 1.11
	10	2.25 ± 0.39 **	10.21 ± 1.11 ns
	30	3.88 ± 0.48 ***	14.79 ± 1.59 ***
	50	5.18 ± 0.69 ***	22.20 ± 2.20 ***
	100	6.68 ± 0.75 ***	25.92 ± 2.38 ***
	Pc	9.68 ± 0.98 ***	34.63 ± 2.89 ***
TiO ₂	Nc	1.14 ± 1.40	4.34 ± 0.82
	10	1.39 ± 1.93 ***	5.38 ± 0.97 *
	30	1.98 ± 2.93 ***	6.57 ± 1.22 ***
	50	2.21 ± 2.88 ***	7.77 ± 1.52 ***
	100	4.06 ± 5.64 ***	9.65 ± 1.71 ***
	Pc	7.02 ± 5.76 ***	15.78 ± 2.61 ***

4.3.4.2 *In vitro* CBMN assay

It was observed the formation of MN after treatment with different ZnO (0, 10, 20, 50, and 100 µg/ml) and TiO₂ concentrations (0, 10, 20, and 50 µg/ml) for 2 hs (Table 4.5). The Chi-squared analysis of the MN frequencies from HEp-2 cells exposed to different ZnO and TiO₂ concentrations revealed a significant increase in induced MN treated with high concentrations of ZnO (50 and 100 µg/ml) or TiO₂ (50 µg/ml) compared to the untreated cell control ($p < 0.05$). However, lower concentrations (< 50 µg/ml) of either ZnO or TiO₂ did not induce significant alterations in MN induction. The MN frequency increased with ZnO concentration in a specific dose dependent manner ($p < 0.05$, Chi-squared test) from an average frequency of 11 MN per 1000 binucleated cells in the control to 28 MN per 1,000 binucleated cells at 100 µg/ml. For TiO₂, the MN frequencies increased in a similar way reaching 31 MN per 1000 binucleated cells at a concentration of 50 µg/ml.

Table 4.5: The effects of ZnO and TiO₂ on HEp-2 cells in the cytokinesis-blocked micronucleus (CBMN) assay. The genetic damage was evaluated for various concentrations setting up two independent cultures per dose. The induction of micronuclei was assessed in a total of 1000 binucleated cells with two nuclei of approximately equal size, while the nuclear division index (NDI) was calculated from a total of 1000 cells (mononucleated, binucleated and multinucleated cells) using the Contingency table. The number of micronuclei was analyzed statistically with the Chi square test.

Treatment concentration in µg/ml		NDI	MN per 1000 BNC
Control	0	2.03	11
ZnO	10	1.68	17
	20	1.81	17
	50	1.62	23 *
	100	1.82	28 **
TiO ₂	10	1.70	13
	20	1.60	19
	50	1.57	31 **
	100	-	Precipitated
MMC	0.134	1.76	49 ***

* $p < 0.05$, ** $p < 0.01$; BNC - binucleated cells; NDI, nuclear division index calculated as described previously; MMC - mitomycin C

4.3.5 Tyrosine phosphorylation

HEp-2 cells were challenged with different concentrations of ZnO (10, 20, 50, and 100 $\mu\text{g/ml}$) and TiO_2 (10, 20, and 50 $\mu\text{g/ml}$) for 4 h, and phosphotyrosine expression was analyzed by Western blotting analysis. The level of tyrosine phosphorylation was up-regulated by ZnO nanoparticles, which induced an increase in phosphorylation of tyrosine residues on 20, 21-22, 25, 30, and ~ 80 kDa proteins (Figures 4.4 and 4.5). Less effect was seen with TiO_2 inducing a fainter effect at 20, 21-22, 25, and 30 kDa at the highest concentrations; however, no effect was observed at ~ 80 kDa.

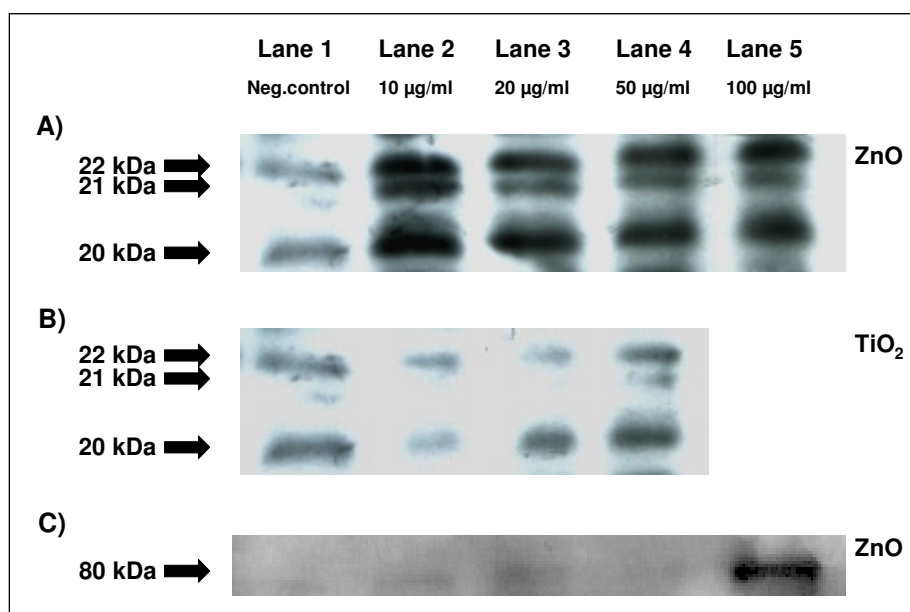


Figure 4.4 Effects of A) and C) ZnO and B) TiO_2 on HEp-2 receptor signalling are shown. An increase in phosphorylation of tyrosine residues can be seen in a Western blot of equal amounts of total proteins from HEp-2 cells treated for 4 hours with different ZnO concentrations 10, 20, 50 and 100 $\mu\text{g/ml}$ (lanes 2 to 5). In lane 1, the result for untreated HEp-2 cells is shown. This figure also illustrates, that two specific bands of increased phosphorylation at 20 kDa and 21-22 kDa were observed in cells treated with A) ZnO (10-100 $\mu\text{g/ml}$) and B) TiO_2 (50 $\mu\text{g/ml}$ only). C) For HEp-2 cells treated with 100 $\mu\text{g/ml}$ ZnO additionally showed an increase in phosphorylation of a protein with a size of 80 kDa.

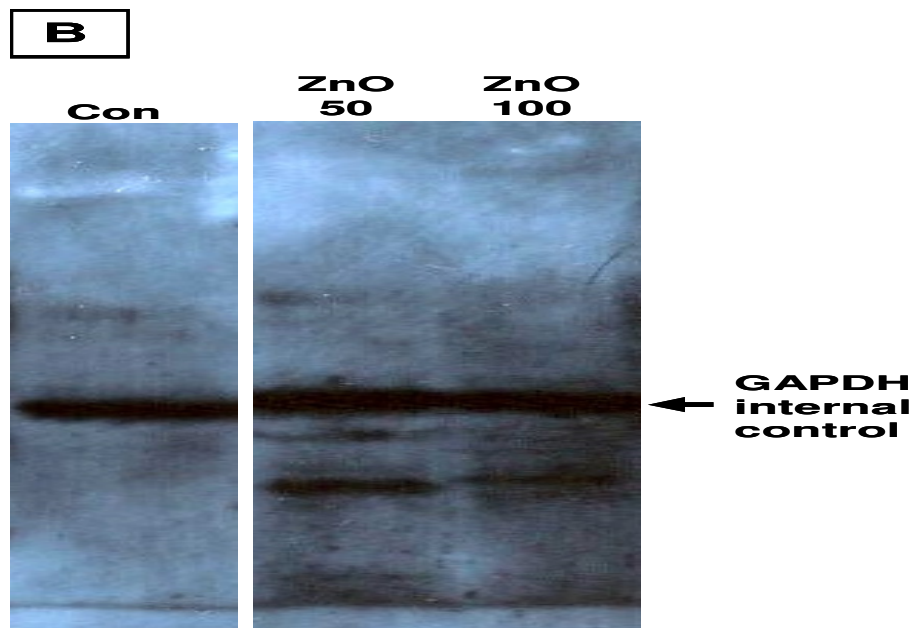
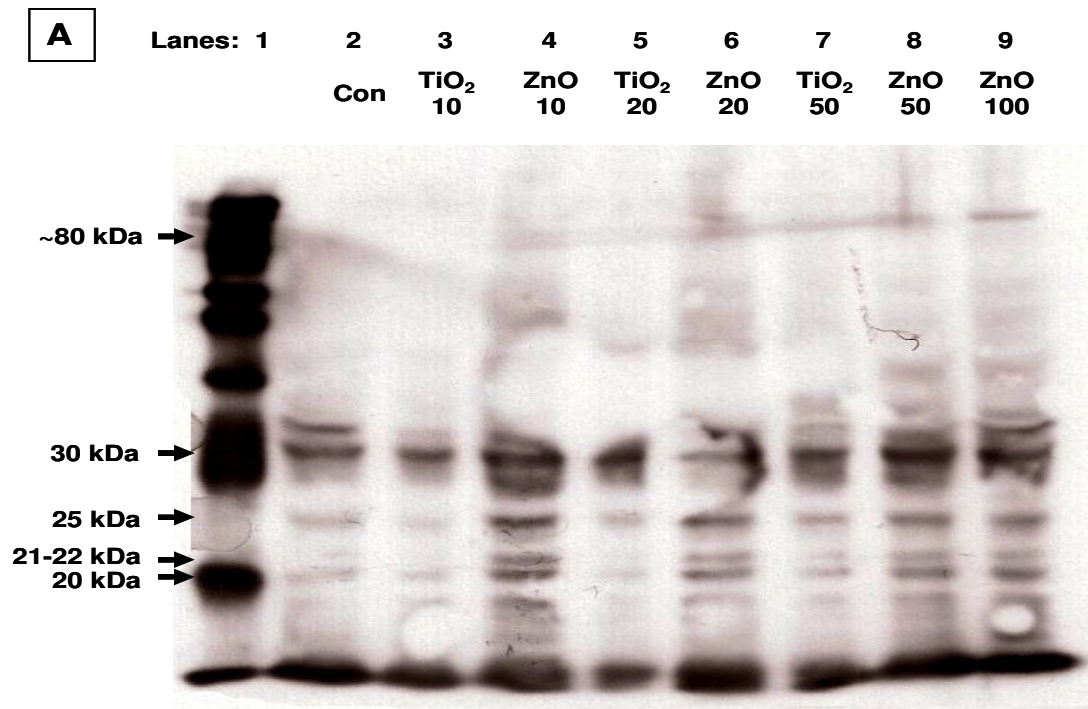


Figure 4.5 Effects of ZnO and TiO₂ on HEP-2 phosphotyrosine receptor signalling are shown. An increase in phosphorylation of tyrosine residues can be seen in a Western blot of equal amounts of total proteins from HEP-2 cells treated for 4 h with different concentrations 10, 20, and 50 µg/ml as well as 100 µg/ml (only for ZnO). Untreated HEP-2 cells served as a negative control (Con). Panel A illustrates for ZnO that all bands at all concentrations are phosphorylated at 20 and 21-22 kDa, but also at 25 and 30 kDa and to some extent at ~80 kDa. For TiO₂, there is less of an effect at the three concentrations examined at 20 and 21-22 kDa. The values represent the mean of three separate experiments. Panel B shows as an example the effect of GAPDH as an internal control seen alongside ZnO at 50 and 100 µg/ml to confirm equal protein loading.

4.4 Discussion

Many toxicology studies over the past years have raised concerns regarding the safety of nanoparticles as they readily generate oxidative stress through reactive oxygen species (ROS). A strong link between nanoparticles and oxidative stress via ROS has been documented (Stone et al., 2007; Xia et al., 2006). Many investigators have shown that ROS play a major role in nanoparticle-induced DNA damage (Donaldson and Stone, 2003). However, detailed molecular mechanisms involved in the cellular responses to genotoxic effects are not yet fully understood. Oxygen radicals can cause single and double strand breaks. Damaging the DNA sugar-phosphate backbone or the bases may then lead to acute DNA damage, which triggers cell cycle arrest and leads to prolonged repair time or cell death (Friedberg and Meira, 2004). Accumulation of mutations which are caused by excessive or incomplete DNA repair is a known cause of oncogenesis (Rusyn et al., 2004). Morz and colleagues found that nanoparticles produced an effect similar to irradiation by inducing carcinogenesis pathways through ROS-induced DNA damage, as well as activating p53 and proteins related to DNA repair (Mroz et al., 2008). The DNA damage may be either primary as a direct effect of the nanoparticles or a secondary effect due to generated ROS (Schins and Knaapen, 2007). Accordingly, ROS may be one of the possible modes suggested for ZnO and TiO₂ induced DNA damage. Oxidative stress is now considered a major cellular signalling regulator and it was suggested that genotoxicity mediated by ROS could be further linked to other pathways (Schins and Knaapen, 2007); therefore, we investigated the tyrosine phosphorylation pathway additionally to the induction of DNA damage.

In view of the importance of tyrosine kinase activity in the control of cell function and the importance of redox mechanisms in regulating the phosphorylation by tyrosine kinases, it was of interest to determine whether the functional changes induced by

altering the redox status of HEp-2 cells were associated with changes in the pattern of tyrosine phosphorylation. The immunoblotting analyses of HEp-2 cells cultured and treated with ZnO for 4 hours, then using a monoclonal anti-phosphotyrosine antibody, revealed heavily phosphorylated tyrosine residues on a protein with a molecular mass of 20 kDa (Figure 4.4 & 4.5). Also bands at 25 and 30 kDa showed phosphorylation of tyrosine residues. A diffuse band of immuno-reactive material could also frequently be observed migrating in advance of this protein with a molecular mass of 21-22 kDa. This could be highly significant as a correlation was previously found between cell transformation and the tyrosine phosphorylation of 22-kDa protein (Glenney, 1989). In addition, previous studies showed, that dysregulation of protein tyrosine phosphorylation is crucial for cell signalling maintenance with subsequent cell proliferation and the early stages of neoplasia (Blume-Jensen and Hunter, 2001; Hahn and Weinberg, 2002). Stimulation of HEp-2 cells with ZnO was also associated with a dramatic enhancement in the intensity of phosphotyrosyl proteins. The increase in a 20 kDa protein tyrosine phosphorylation induced by ZnO was observed over the whole range of concentrations (10-100 µg/ml). Zinc oxide nanoparticles at a dose of 100 µg/ml additionally had a stimulatory effect on tyrosine phosphorylation of an 80 kDa protein (Figure 4.3 & 4.4). The same dose gave a significant 3-fold increase in the Olive tail moment and % tail DNA in the Comet assay when compared to the untreated control (Table 4.4). On the other hand, TiO₂ produced fainter bands at 20 kDa and only for the 50 µg/ml treatment dose phosphorylation at 21-22, 25, and 30 kDa (Figure 4.4 & 4.5). No effect was seen at ~80 kDa for TiO₂. As a result, tyrosine phosphorylation may have contributed to the genotoxicity of ZnO but less to TiO₂ genotoxicity.

The consequences of genotoxic injury to HEp-2 cells were examined by ZnO and TiO₂ to elaborate the signalling events that modulate cell survival after genotoxic exposure.

Previous studies on HeLa cells showed activation of Cdk1 as a result of etoposide treatment lead to cell cycle checkpoint override with subsequent cell death (Jiang et al., 2005). In *in-vitro* studies on human U937 monocytes nano-cobalt increased the transcription of matrix metalloproteinases (MMP-2 and MMP-9) and protein tyrosine kinase (PTK) signalling pathways were involved through oxidative stress (Wan et al., 2008). Additionally, sodium vanadate was also found to induce protein tyrosine phosphatase (PTP) inhibition. This resulted in a bypass of growth arrest that occurred as the result of genotoxic exposure which indicates a role for tyrosine phosphorylation in the regulation of cell survival (Bae et al., 2009). PTP inhibition was also previously reported in primary mammary epithelial cells which enhanced cell survival by decreasing apoptosis (Furlong et al., 2005).

In this study, cytotoxic and genotoxic effects of ZnO and TiO₂ were tested in HEp-2 cells. Cytotoxicity was measured using the MTT and NRU assays; genotoxicity was assessed by the Comet and CBMN assays to measure the DNA damaging and genotoxic potential. The data showed a cytotoxic effect of ZnO in mitochondrial activity (MTT assay) and the ability of lysosomes to retain neutral red stain (NRU assay) in HEp-2 cells after treatment for 24 and 48 hours with different ZnO concentrations with the exception of the lowest dose (10 µg/ml) which showed no cytotoxicity even after 48 hours treatment (Figure 4.2, Table 4.2 and 4.3). The treatment times used for cell cultures extended well beyond the 4 h treatment times used for the Comet assay. On the other hand, 100 µg/ml of TiO₂ proved to be significantly toxic after 2 hours on HEp-2 cells (Figure 4.2). For both doses, 50 and 100 µg/ml, TiO₂ precipitated in the wells after 24 hours. Hence, treated cells showed an OD which was > 140% when compared to the untreated control in the MTT and NRU assays. The TiO₂ concentration of 20 µg/ml was cytotoxic to the cells in both assays to different extents in 24 hours treatment assays.

Aggregation of nanoparticles observed in our study has been reported previously for TiO₂ (Lovern and Klaper, 2006) as well as for ZnO (Adams et al., 2006b) and is considered as an inherent property of uncoated oxide nanoparticles which occur in aqueous conditions from distilled water to complex biological media. Although, previous studies have shown that particle solubility of metal oxide nanoparticles like ZnO which release free hydrated zinc ions (Zn²⁺) strongly influenced cytotoxicity compared to extremely less soluble metal oxides like TiO₂ (Brunner et al., 2006; Franklin et al., 2007; Xia et al., 2008), in this study, as the particle size increased with the concentration, the aggregation did not change over a time period of up to 48 hours for ZnO and 24 hours for TiO₂. Despite increasing in ZnO and TiO₂ sizes, particles stayed well below the pharmaceutical size definition (< 1000 nm) for nanoparticles (Sun et al., 2004; Teeguarden et al., 2007). Therefore, it was assumed that the rate and quantities of dissolution was either negligible or of little significance for the experiments.

The Comet assay results demonstrated the DNA damaging potential of different concentrations of ZnO or TiO₂ with significant increases in the tail moment and % tail DNA in the Comet assays (3-fold for ZnO) at the highest concentration and (2-fold for TiO₂) at the highest concentration compared to untreated control (Figure 4.3). The DNA damaging and mutagenic potential of ZnO or TiO₂ at various concentrations was also seen for the CBMN assays. The genotoxic potential of ZnO or TiO₂ is supported by a previous studies conducted by Gopalan and colleagues (Gopalan et al., 2009), which showed a concentration-related increase in Olive tail moment in lymphocytes and sperm. Also, an increase in chromosome aberrations in CHO cells treated with ZnO has been previously reported (Dufour et al., 2006). PTP inhibition (Bae et al., 2009) or

increased transcription of matrix metalloproteinases (MMP-2 and MMP-9) and PTK signalling pathways (Wan et al., 2008) could explain our findings of increased tyrosine phosphorylation as the result of genotoxic insult of ZnO and the highest concentration of TiO₂.

4.5 Conclusions

The present investigation conclusively provides further evidence for the genotoxic effect of ZnO and TiO₂ nanoparticles in both the Comet assay and the micronucleus assay with induction of chromosomal damage at high, but non-cytotoxic (according to Trypan blue exclusion) concentrations of ZnO (50 and 100 µg/ml) and TiO₂ (50 µg/ml). It also provides evidence of significantly increased chromosomal mutations at the highest concentrations which showed no cytotoxic effects in the NRU and MTT assays. Furthermore, ZnO increased tyrosine phosphorylation of five proteins at 20, 21-22, 25, 30, and ~80 kDa with less effect of TiO₂ which showed increased phosphorylation compared to the negative control at 20, 21-22, 25, and 30 kDa for the highest dose but not at ~80 kDa. Thus, tyrosine phosphorylation is modified by both ZnO and TiO₂ while causing genotoxic damage seen for two different end points in the Comet and CBMN assays in the absence of cytotoxicity.

4.6 Executive summary

- Cytotoxicity of ZnO

The mitochondria and lysosome activity tests (MTT assay and NRU) indicated cytotoxicity of ZnO to HEp-2 cells treated beyond 4 h reaching cell survival of < 16% after 48 hours.

- Cytotoxicity of TiO₂

The highest TiO₂ concentration (100 µg/ml) was cytotoxic to HEp-2 cells in less than 2 hours.

- Genotoxicity of ZnO

Genotoxicity of ZnO in HEp-2 cells was seen within the Comet assay showing a 3-fold increase in DNA damage. For the CBMN assay, an increase in the formation of micronuclei was found after treatment with different concentrations ZnO.

- Genotoxicity of TiO₂

The genotoxic potential of TiO₂ in HEp-2 cells was confirmed in both cytogenetic assays, for the Comet assay showing a 2-fold increase in DNA damage after treatment and for the CBMN assay revealing a dose-dependent increase in MN formation.

- Tyrosine phosphorylation of HEp-2 cells treated with ZnO

The level of tyrosine phosphorylation was up-regulated after exposure to ZnO nanoparticles, significantly increasing phosphorylation events of tyrosine residues on 20 kDa, 21-22 kDa, 25 kDa, 30 kDa, and 80 kDa proteins.

- Tyrosine phosphorylation of HEp-2 cells treated with TiO₂

TiO₂ showed less effect on tyrosine phosphorylation at the higher doses. There was some effect on 21-22, 25, and 30 kDa proteins but not at ~80 kDa.

Chapter 5

A comparison of the effects of titanium dioxide (TiO₂) nanoparticles in peripheral blood lymphocytes from respiratory disease patients and healthy individuals using genotoxicity endpoints and ras p21

5.1 Introduction

Nanotechnology has great potential in medical and biomedical applications and is increasingly used in a variety of applications such as prostheses, implants, medical imaging as well as in drug-delivery devices, which have been used for drug targeting of specific sites such as cancer sites and for gene delivery (Colilla et al., 2008; Kim et al., 2008; Wan et al., 2007; Wang et al., 2008). In addition, NPs have been used for treating many human diseases such as tumours due to their unique physicochemical properties which have helped in the production of many nanomedicines, some currently in use and many under development, which bring human cells into direct contact with the NPs (Arvizo et al., 2010; Beck-Broichsitter et al., 2010; Ossipov, 2010).

Titanium dioxide, TiO_2 , has well known photo-catalytic properties, hence it was used as a photosensitizer in photodynamic therapy for bronchial and oesophageal cancer treatment (Ackroyd et al., 2001). Substantial numbers of reports have shown that, ultraviolet (UV) light can cause skin damage, aging and cancer. Hence due to the TiO_2 property of effectively blocking long waves of ultraviolet sun light from 400–700 nm wavelengths (Maier and Korting, 2005), it is widely used in sun creams and cosmetics as a sun blocking factor (SBF) to shield and protect human skin from the dangers of UV light (Antoniou et al., 2008). TiO_2 is also routinely applied to waste water and released to the environment as a disinfectant (Cho et al., 2004). In addition, TiO_2 crystalline forms (anatase and rutile) absorb UV light in aqueous media hence, catalyzing the generation of different reactive oxygen species, such as free hydroxyl radicals, hydrogen peroxide and superoxide anion radicals (Hirakawa et al., 2004; Kawahara et al., 2003).

The industrial use of titanium dioxide is rapidly increasing in a variety of everyday life applications such as papers, plastics and sun creams. In addition, TiO_2 is frequently used in paints because of its whiteness and brightness and as the consequence of such wide use; the environmental and occupational exposure to TiO_2 is consequently increasing, with the information regarding the hazardous effects to human health exposed to TiO_2 dust lagging behind. Several studies have shown that nanoparticles may be more toxic than larger particles of the same substance because of the NPs large surface area, which could enhance NPs chemical reactivity and subsequently make it easier to penetrate the cell (Lam et al., 2004).

The effect of titanium dioxide (TiO_2) of a size greater than 100 nm on humans and animals was previously classified as being biologically inert (Hart and Hesterberg, 1998; Lindenschmidt et al., 1990), as a result it has been increasingly used as an additive in many consumers products such as food colourant (Lomer et al., 2002), white pigment, and in sunscreens and cosmetic creams (Gelis et al., 2003). However, other studies have shown that the NPs size was not the effective factor of their cytotoxicity (Yamamoto et al., 2004).

Although the previous studies have shown that the pathological effects of TiO_2 dust to the lungs is not as great as asbestos and silica particles, still some consideration has been given to its health effect as nuisance dust (Becker et al., 2002; Chen et al., 2009; Driscoll et al., 1997; Schins, 2002). Garabrant and colleagues (1987) have found that 17% of the workers exposed to TiO_2 metal production developed pleural thickening and plaques on their X-rays and the extent of the thickening and plaques was related to the duration of work in TiO_2 manufacturing environment. In addition, TiO_2 dust exposure has also been reported to deposit in the lung tissues and induce lung fibrosis (Bermudez

et al., 2004; Lee et al., 1985). Studies on in alveolar type II cells have shown that TiO₂ as well as other poorly soluble particles such as carbon black and quartz increased *hprt* gene mutation (Driscoll et al., 1997; Schins, 2002)

In vivo studies in animals have shown that rat lungs instilled with low doses of commonly used TiO₂ did develop pulmonary inflammation or cytotoxicity (Rehn et al., 2003; Warheit et al., 2005). Numerous studies have shown that TiO₂ exposure significantly increases the production of hydroxyl radicals (Lee et al., 1985; Rossi et al., 2010) but the cytotoxicity of particular TiO₂ was low compared to other nanoparticles (Peters et al., 2004; Yamamoto et al., 2004).

The ability of TiO₂ to cause inflammation or to produce inflammatory cells was also tested, as for example, *in vivo* and *in vitro* administration of TiO₂ have shown an increase in the activity of growth factors or the release of tumour necrosis factor secreted from macrophages (Renwick et al., 2001; Schins, 2002). Studies of TiO₂ on Chinese hamster ovary (CHO-K1) cells, was shown to enhance MN production which was dose-dependent (Lu et al., 1998). On the other hand, *in vitro* assays on the same cells (CHO), has found that TiO₂ did not induce MN possibly due to poor solubility of TiO₂ (Miller et al., 1995) .

In vivo studies on mice have reported that TiO₂ NPs induced 8-hydroxy-2'-deoxyguanosine, gamma-H2AX foci, micronuclei, and DNA deletions. Titanium dioxide/hexachloroethane smoke condensate has been reported to induce mutations in strains TA98 and TA100 of *Salmonella* assays, inhibited erythropoietic activity but did not induce micronuclei in mice (Karlsson et al., 1991). However, in a separate report mice exposed to TiO₂ three times a day have induced significant elevation in MN frequency (Shelby and Witt, 1995).

Titanium dioxide without photoactivation has been shown not to induce DNA damage in human cells (Dunford et al., 1997) or genotoxicity in rats (Rehn et al., 2003). In contrast to the above mentioned findings, other studies have reported that TiO₂ NPs tested without photoactivation, caused a significant dose-dependent increase in micronuclei production and apoptosis in Syrian hamster embryo fibroblasts (Rahman et al., 2002) and chronic pulmonary inflammation in rats (Oberdorster et al., 1992).

Given the scale of nanotechnology production, therefore, it is inevitable that the waste of nanotechnology products will accumulate and contaminate the environment. It is necessary to understand how engineered TiO₂ NPs interact with human cells following exposure of the more vulnerable respiratory disease patients compared to the healthy individuals. Lymphocytes from pulmonary disease patients and controls were used as a surrogate for respiratory cells to test whether TiO₂ is genotoxic in the Comet and CBMN assays and to determine whether the respiratory disease patients had a specific mechanism, which could contribute to TiO₂ potential toxicity. The respiratory pathway is the main route of entry of environmental pollutant particles to the body through inhalation. Accordingly, this study was to determine if there are any differences in sensitivity to nano-chemical insult (TiO₂) between the patient and control groups.

Nanotechnology has raced ahead of nanotoxicology and little is known of the effects of nanoparticles in human systems, let alone in diseased individuals. This study was carried out to investigate the health effect of TiO₂ on patients with respiratory diseases (lung cancer, COPD and asthma) compared to healthy individuals because their respiratory system is compromised by their primary lung disease. In addition, previous studies have shown that oxidative stress, induced by exposure to hydrogen peroxide, can produce differences in response between respiratory disease patients with lung

cancer, COPD and asthma and a healthy control group, and between different patient groups (Falque-Gonzales, 2008). Confounding factors such as age and gender and smoking have been taken into account. Responses have been measured using different end-points for genotoxic effects. These include the Comet assay, the micronucleus assay and ras oncoprotein level determinations. Therefore, our objective was to compare the effects of nanoparticles in peripheral blood lymphocytes from patients with respiratory diseases and healthy individuals using genotoxic endpoints. Thus, in this report the genotoxic effects of anatase TiO₂ were examined in the absence of UV irradiation (Comet assays and CBMN) and on the ras p21 signalling pathway on lymphocyte cells from respiratory disease patients as well as from healthy control individuals.

5.2. Materials and Methods

For materials used in each experiment see table 2.1

5.2.1.1 Blood samples

This study was approved by Leeds (Central) Research Ethics Committee (REC reference number: 09/H1313/37) and the Research Support & Governance Office, Bradford Teaching Hospitals NHS Foundation (ReDA number: 1202). Ethical permission was also provided by University of Bradford Research Ethics sub Committee on Research in Human Subjects reference no: 0405-8.

5.2.1.1 Patients samples

The patients for this study were from the Outpatient Respiratory Disease Clinic of Dr. Badie K. Jacobs (St Luke's Hospital, Bradford, UK). The criteria for cases included lung cancer without prior chemotherapy or radiotherapy, COPD or asthma. Exclusion criteria included anaemia, other diseases beside their respiratory condition or previous occupational exposure to other NPs (e.g. silica or asbestos). All individuals were given

an information sheet, completed an interviewer questionnaire and signed a consent form prior to 36 ml of blood being taken from each patient in heparanized tubes (9 ml in each of 4 tubes). The population for this study consisted of 51 subjects in total, 36 respiratory disease patients and 15 healthy controls. The sub group of the respiratory disease persons was as follows: 16 patients with lung cancer, 11 with COPD and 9 with asthma. According to gender, the population included 19 males and 17 females with respiratory diseases and 10 males and 5 females as healthy controls. The patients were aged 6 below 50, 14 aged 50-65 and 16 above 65 years of age, the controls aged 9 < 50 and 6 aged 50-65. As for smoking habits, 28 smoking patients who smoked an average of 18 cigarettes per day over an average of 25 years and 8 non present smokers while all controls were non smokers. Regarding ethnicity, the subjects for this study have been detailed in table 5.1. This study has been carried out as a coded study, as the investigator had no prior knowledge of the diagnosis of each sample used.

5.2.1.2 Healthy controls

The individuals for this study were recruited from healthy controls at Bradford University. All individuals were given an information sheet, completed an interviewer questionnaire and signed a consent form prior to 36 ml of blood being taken from each patient in heparanized tubes. Exclusion criteria included anaemia, chest disease or any other disease. Healthy control individuals used in this study have also been detailed in table 5.1.

Table 5.1: Demography of the respiratory disease patient and healthy control populations: includes, respiratory disease patients (lung cancer, COPD and Asthma) coded as P and healthy control volunteers coded as V. Numbers indicate the sequence in which samples were received. The total of 36 patients and 15 healthy controls samples were used in the Comet and CBMN assays with the exception of ^x (denotes not used in CBMN assay).

Serial No.	Study code	Age	Gender	Ethnicity	Smoking history	Cigarettes per day	Years smoked	Diagnosis
1	P 1	69	M	Caucasian	Past	30	50	lung cancer ^x
2	P 13	60	M	Caucasian	yes	20	15	lung cancer ^x
3	P 14	72	M	Caucasian	yes	20-30	39	lung cancer
4	P 15	77	M	Caucasian	yes	15	30	lung cancer
5	P 18	58	M	Caucasian	No	-	-	lung cancer
6	P 20	51	F	Caucasian	yes	20	30	lung cancer
7	P 22	69	F	Caucasian	yes	20	50	lung cancer
8	P 24	81	M	Caucasian	yes	15	40	lung cancer
9	P 25	70	M	Caucasian	yes	20	50	lung cancer
10	P 27	64	M	Caucasian	yes	15-20	50	lung cancer
11	P 28	70	F	Caucasian	yes	20	50	lung cancer
12	P 30	81	M	Asian	yes	20	34	lung cancer
13	P 31	63	F	Caucasian	yes	20	30	lung cancer
14	P 32	78	M	Caucasian	yes	20-30	60	lung cancer
15	P 33	73	F	Caucasian	yes	15	15	lung cancer ^x
16	P 35	64	F	Caucasian	yes	20	50	lung cancer
17	P 2	62	M	Caucasian	Past	15-20	20	COPD
18	P 3	74	F	Caucasian	yes	15	50	COPD
19	P 4		F	Caucasian	yes	20	30	COPD
20	P 7	76	F	Caucasian	Passive	-	-	COPD
21	P 10	72	M	Caucasian	No	-	-	COPD
22	P 11	73	M	Caucasian	pipe	2 oz/wk		COPD ^x
23	P 12	64	M	Caucasian	yes	10	20	COPD
24	P 16	69	F	Caucasian	yes	10	40	COPD
25	P 21	63	M	Caucasian	yes	20	40	COPD
26	P 23	62	F	Caucasian	Past	10-20	40	COPD
27	P 29	69	F	Caucasian	yes	20	40	COPD
28	P 34	53	M	Caucasian	yes	20	35	Asthma
29	P 5	44	F	Asian	yes			Asthma
30	P 6	28	F	Asian	No	-	-	Asthma
31	P 8	47	F	Kenyan	yes	12	20	Asthma
32	P 9	58	M	Asian	No	-	-	Asthma
33	P 17	56	F	Asian	No			Asthma
34	P 19	63	F	Caucasian	Past	30	10	Asthma ^x
35	P26	41	F	Asian	No	-	-	Asthma
36	36	29	F	Caucasian	yes	6	7	Asthma
37	V1	34	M	Asian	No	-	-	Healthy control
38	V2	29	M	Caucasian	No	-	-	Healthy control
39	V3	29	M	Asian	No	-	-	Healthy control
40	V4	29	M	Caucasian	past	-	-	Healthy control
41	V5	51	M	Caucasian	No	-	-	Healthy control
42	V6	54	F	Caucasian	No	-	-	Healthy control
43	V7	63	M	Asian	No	-	-	Healthy control
44	V8	31	F	Caucasian	No	-	-	Healthy control
45	V9	57	F	Caucasian	No	-	-	Healthy control
46	V10	54	M	Asian	No	-	-	Healthy control
47	V11	45	F	Asian	No	-	-	Healthy control
48	V12	52	F	Caucasian	No	-	-	Healthy control
49	V13	67	M	Caucasian	No	-	-	Healthy control
50	V14	55	M	Caucasian	No	-	-	Healthy control
51	V15	37	M	Caucasian	No	-	-	Healthy control

Table 5.2: Demography of the population used for ras p21 studies: includes, respiratory disease patients (lung cancer, COPD and Asthma) coded as P and healthy control volunteers coded as V.

Study code	Age	Gender	Ethnicity	Smoking history	Cigarettes per day	Years smoked	Diagnosis
V1	34	M	Asian	No	-	-	Healthy control
V5	51	M	Caucasian	No	-	-	Healthy control
V6	54	F	Caucasian	No	-	-	Healthy control
V9	57	F	Caucasian	No	-	-	Healthy control
V11	45	F	Asian	No	-	-	Healthy control
V13	67	M	Caucasian	No	-	-	Healthy control
P 1	69	M	Caucasian	Past	30	50	lung cancer
P 13	60	M	Caucasian	yes	20	15	lung cancer
P 14	72	M	Caucasian	Yes	20-30	39	lung cancer
P 15	77	M	Caucasian	yes	15	30	lung cancer
P 18	58	M	Caucasian	No	-	-	lung cancer
P 20	51	F	Caucasian	Yes	20	30	lung cancer
P 22	69	F	Caucasian	Yes	20	50	lung cancer
P28	70	F	Caucasian	yes	20	50	lung cancer
P 2	62	M	Caucasian	Past	15-20	20	COPD
P 3	74	F	Caucasian	Yes	15	50	COPD
P 7	76	F	Caucasian	Passive	-	-	COPD
P 10	72	M	Caucasian	No	-	-	COPD
P 11	73	M	Caucasian	Pipe	2 oz/wk		COPD
P 12	64	M	Caucasian	Yes	10	20	COPD
P 16	69	F	Caucasian	Yes	10	40	COPD
P 21	63	M	Caucasian	Yes	20	40	COPD
P 34	53	M	Caucasian	Yes	20	35	Asthma
P 5	44	F	Asian	Yes			Asthma
P 6	28	F	Asian	No	-	-	Asthma
P 17	56	F	Asian	No			Asthma
P 34	53	M	Caucasian	Yes	20	35	Asthma

5.2 Methods

5.2.2.1 Experimental design

The experiment was designed to find the difference in response of lymphocytes exposed to different concentrations of TiO₂ NPs, between respiratory disease patients with lung cancer, chronic obstructive pulmonary disease (COPD), asthma and a healthy control group, and between different patient groups. Confounding factors such as age and gender and smoking habit have been taken into account. Responses were measured using different end-points for genotoxic effects. These involved the Comet assay, the micronucleus assay and ras oncoprotein level determinations.

5.2.2.2 Comet assay

The Comet assay measures DNA strand breakage in any single cell (Tice *et al*, 2000). Briefly in the Comet assay, cells can be taken from humans (human blood) and treated with a chemical or series of chemicals to determine effects on the genome. Cells are distributed on slides in an agar ‘sandwich’. The DNA is lysed and slides placed in an electrophoresis buffer. Then broken DNA strands move towards the anode forming a tail which has the appearance of a Comet. The greater the tail length and width, the greater the extent of DNA damage. Depending on the pH of the electrophoresis buffer, single or double strand breaks can be measured. Cells can be examined immediately after treatment, or left for some time to allow DNA repair to take place. Damage was measured with the Kinetic Image Analysis system using KOMET 4 software.

5.2.2.2.1 Chemical preparation

Different concentrations of TiO₂ NPs suspensions (10, 30 and 50 µg/ml) were prepared through sonication then, particle size was measured by a nano-sizer, 80 µM (2.72 µg/ml) of H₂O₂ was also prepared as a positive control (Pc) (detailed in section 2.2.1.1).

5.2.2.2.2 Treatment of lymphocytes

In this *ex vivo/in vitro* study of DNA damage and repair in lymphocytes from healthy and lung disease patients, the lymphocytes were isolated according to the protocol (detailed in section 2.2.1.2) within 2 hours of blood extraction. The lymphocytes were then treated with different concentrations of TiO₂ (10, 30 and 50 µg/ml) as well as a negative control of untreated lymphocytes (Nc) and 80 µM (2.72 µg/ml) of H₂O₂ (Pc) then incubated for 30 minutes at 37°C. The Comet assay was then carried out using the Comet pH ≥ 13 system (detailed in section 2.2.2).

5.2.2.3 Cytokinesis-block micronucleus assay

Cytogenetic damage can be measured as micronuclei in cells after chemical treatment (Fenech, 2007). The greater the number of micronuclei, the greater the effect on the cell genome. Cells have a fixed chromosome number depending on the species and man has 46. A small amount of blood can be used to make the micronucleus preparations. A brief general description of the method as follows: a blood culture was prepared from each (coded) sample in 25 cm³ cell culture flasks, each culture consisted of 500 µl of whole blood added to 4.5 ml fresh medium of RPMI 1640 with Glutamax-I which contains 20% fetal bovine serum (Gibco) and 1% penicillin-streptomycin solution. (P/S), and this fresh medium allows the cells to grow. 130 µl of phytohaemagglutinin (PHA), a mitogen, was added to reach a final concentration of 2.5%, added because lymphocytes need to be stimulated to divide and proliferate. Cultures were then left in an incubator whilst the chemicals 50 µl of TiO₂ at 2 different concentrations (5 and 10 µg/ml) and 50 µl of MMC were added as a positive control to reach a final concentration of 0.4 µM (Meynard et al., 2007) at time 24 hours. Cytochalasin B at a final concentration of 6 µg/ml was added to cell cultures at 44 hours which prevents the

cells undergoing cytokinesis, so that binucleated cells are formed (Fenech, 2007). Incubation is continued until 72 hours. They were fixed with a 3:1 methanol: acetic acid fixative and stained with Giemsa. Each sample was treated in duplicate flasks with TiO₂ as well as positive and negative controls.

Two slides were prepared from each culture (a total of 4 slides per sample). Micronuclei were counted using a 100X oil immersion objective on a light microscope. A total of 31 patients (13 lung cancer, 10 COPD and 8 asthma) and 15 controls were used in the CBMN assay; 5 samples were excluded for technical reasons (refer to section 2.2.3.7).

5.2.2.4 Immunoblotting for ras oncoprotein detection

In general, a standard amount of protein from lymphocyte culture lysates, that were obtained from 20 chest disease patients and 6 healthy control blood samples was quantitated using the Bio- Rad *DC* method and separated by gel electrophoresis, transferred to a nitro-cellulose membrane by Western blotting and detected by chemiluminescence (Anderson et al., 1998a). A monoclonal ras antibody was used as a primary antibody and horse-radish peroxidase-conjugated sheep anti-mouse immunoglobulin as the secondary antibody. Optical densities (OD) of the peak of the protein bands were calculated and values of two standard deviations above negative control means were considered positive. A pan-ras antibody, which can detect proteins produced by all three members (Harvey, Kirsten and N- ras) of the *ras* gene family was used. Lymphocyte cultures and protein extraction method are detailed in chapter 2 section (2.2.3.4, 2.2.2.7.3). For ras p21 detection; the individuals whose lymphocytes used were shown on table (5.2). In brief 6 healthy controls, 4 asthma, 8 COPD and 8 lung cancers were used in this study.

5.2.2.5 Statistics

For the Comet assay the Olive tail moment (OTM) and % tail DNA parameters were used in the treated and untreated lymphocytes in addition to the negative (Nc) and the positive control H₂O₂ of the respiratory disease patient groups and healthy control groups. The Olive tail moment (OTM) and % tail DNA parameters have been used in this study as the use of two different Comet parameters was recommended (Kumaravel et al., 2009). The results shown are the means of two independent experiments per patient or healthy control. One hundred cells were scored in each experiment to enhance the statistical power. SPSS 15 for windows was used. The Kolgomrov-Smirnov was used to examine the OTM and % tail DNA data for normality of distribution. The data was found to have a normal distribution and accordingly, the parametric ANOVA test was applied. Groups were compared in 2 different ways; in the first each group was compared to its own untreated control and in the second each patient group or confounder was compared to its parallel healthy control sample.

For CBMN the nuclear division index (NDI) can be used as an indicator of the cytostatic effect of TiO₂ and it was calculated according to the formula: $NDI = (M_1 + 2M_2 + 3M_3) / N$; where M_1 , M_2 and M_3 presented mono-nucleated, bi-nucleated and multi-nucleated cells respectively while N represented the total viable cells scored. The nuclear division index (NDI) was tested with the Chi square (χ^2), and the number of MN in 1000 binucleated cells was compared between the untreated and treated for each sample using the graph pad 2x2 contingency (Fisher exact test) for the *P* value. For ras p21 immunoblotting Image J software for windows was used to quantify western blots to compare the OD of the bands (Chiang et al., 2009; Neiryneck et al., 1991).

5.3 Results

5.3.1 Comet assay in lymphocytes from respiratory disease patients and controls:

Groups were compared in 2 different ways; in the first, each group was compared to its own untreated control and in the second each patient group or confounder was compared to its parallel healthy control sample. The Olive tail moment (OTM) and % tail DNA of the healthy controls and patient group lymphocytes treated with different TiO_2 concentrations were then analyzed by ANOVA and the following results obtained: table 5.3 and 5.4 and figures 5.1(A-E): In all reported experiments the positive control, H_2O_2 , was always statistically significant $p > 0.001$. This indicated that the system and whole experiment had worked satisfactory.

5.3.1.1 Patient and control groups

In figure 5.1 A and tables 5.3 & 5.4, the means of the OTM and % tail DNA showed that TiO_2 produced genomic damage which increased in a concentration dependent manners in both respiratory disease patients and controls lymphocyte cells. When OTM data were used to compare the whole patient group and the control group, and TiO_2 treated lymphocytes to the untreated lymphocyte control, results showed that TiO_2 increased in a concentration dependent way DNA damage in both groups to varying extent with the exception of the lowest TiO_2 concentration (10 $\mu\text{g/ml}$) which did not induce significant damage in healthy controls (ns). However, the patient group had more DNA damage ($p > 0.001$) with exception of 10 $\mu\text{g/ml}$ of TiO_2 that caused less significant damage to patient lymphocytes with ($p < 0.05$). Moreover, with the % tail DNA diameter, all control samples treated with different TiO_2 concentrations were not statistically significant (ns), and for the patient group only TiO_2 at a concentration of 50 $\mu\text{g/ml}$ was significant ($p < 0.01$).

When the patient groups compared to the control group (table 5.3 & 5.4) the means seen in fig 5.1.A generally showed that, the respiratory disease patient groups had a higher basal DNA damage. This was confirmed when the two untreated controls groups were compared with each other ($p < 0.01$). When comparing each patient's sample with its parallel control sample, patient samples showed statistically significant higher DNA damage for both OTM and % tail DNA parameters simultaneously. The statistical significance for the negative control (Nc) versus TiO_2 10 $\mu\text{g/ml}$ was ($p < 0.01$) and versus TiO_2 concentrations of 30 and 50 $\mu\text{g/ml}$ the significance was at $p < 0.001$.

5.3.1.2 Analysis of confounding factors:

5.3.1.2.1 Lung cancer, COPD, asthma and healthy controls

Figure 5.1B shows the lung disease patients namely lung cancer, COPD and asthma compared to the healthy control group. The trend of the histogram shows more DNA breakage in lung cancer and COPD groups compared to the asthma group of patients and healthy controls. On comparing the OTM of each group of lymphocytes treated with different TiO_2 concentrations to untreated lymphocyte controls by ANOVA, lung cancer and COPD groups showed identical as well as higher DNA damage ($p < 0.001$) at all TiO_2 concentrations with exception of the lowest concentration ($p < 0.05$), while the asthma group showed less damage than the controls and DNA breakage occurred only at the high concentration of 50 $\mu\text{g/ml}$ ($p < 0.001$). The OTM controls group showed significant DNA breakage ($p < 0.01$) but less than in COPD and lung cancer patient. However, % tail DNA of both lung cancer and COPD recorded less DNA damage than on OTM and in both cases and 10 $\mu\text{g/ml}$ did not induce any DNA damage to both. However asthma patients showed the % DNA statistical significance similar to

COPD ($p < 0.05$) and , with the % tail DNA parameter all control samples treated with different TiO₂ concentrations were not statistically significant (ns).

When each of the patient groups (lung cancer, COPD and asthma) were compared to their equivalent control group sample (table 5.3 & 5.4), the COPD group showed higher basal DNA damage at ($p < 0.001$) followed by lung cancer at ($p < 0.01$) while asthma group showed no difference in basal damage compared to the control for both OTM and % tail DNA. All groups showed identical significance for OTM and % tail DNA with exception of asthma which showed less damage with the % tail DNA parameter.

5.3.1.2.2 Gender

Histogram 5.1C and tables 5.3 and 5.4 shows that when the data for each group of treated samples were compared to data for untreated samples in the same group, the following result was reported: The highest DNA damage was for OTM of male patients compared to other groups. Male controls and female patients and female controls showed lesser value for OTM. However the % tail DNA for the male and female subgroups showed that female patients had the highest DNA damage. No damage was induced by TiO₂ in both male and female controls DNA when % tail DNA was examined. However when male and female patient groups were compared to male and female controls (table 5.3 & 5.4), the male patient group had higher damage than female patients with the exception of TiO₂ lowest concentration where there was more genotoxic damage to female DNA ($p < 0.001$). Male and female patients showed identical results for OTM and % tail DNA.

5.3.1.2.3 Age

In figure 5.1D and tables 5.3& 5.4, TiO₂ treated lymphocytes showed increasing damage with age for OTM with patients over 65 expressing the highest DNA damage

followed by patients of 50-65 then the other groups. However for % tail DNA controls 50-65 showed higher damage than patients over 65 and only the highest concentrations caused DNA damage to the patients over 65 ($p < 0.05$), controls less than fifty expressed no DNA damage for % tail DNA. However, patients less than fifty were the only group that had damage ($p < 0.001$) when their lymphocytes were treated with TiO_2 (50 $\mu\text{g/ml}$). When the three patient groups compared to the two controls, the two oldest patient groups expressed similar results except that the over 65 had higher basal damage and patients < 50 had no significant basal damage reported also; the lower dose was not genotoxic to patients < 50 .

5.3.1.2.4 Smoking habit

Figure 5.1 E and tables 5.3 and 5.4 demonstrate that all lymphocytes of smoker's and non smoker's categories showed an upward trend in DNA damage that indicates an increasing genotoxicity in lymphocytes treated with different TiO_2 concentrations with the smoking patients showing the greatest DNA damage which was identical in both Comet parameters and the non smoking controls had the lowest damage with the non smoking patients in between. When the Comet data for smoking and non smoking patients were compared to their corresponding non smoking controls, the results showed that for both Comet parameters, used in our statistical analysis, non smoking and smoking patients had similar baseline damage however, smokers developed more DNA damage after TiO_2 exposure.

5.3.1.2.5 Ethnicity

Figure 5.1F and tables 5.3 & 5.4 demonstrated statistically significant differences between the Asian and Caucasian populations. The Asian patients show statistically significant less damage than the Caucasians ($P < 0.05$). The Caucasian controls do not

differ from the Asian controls. Generally, there was agreement between OTM and % tail DNA.

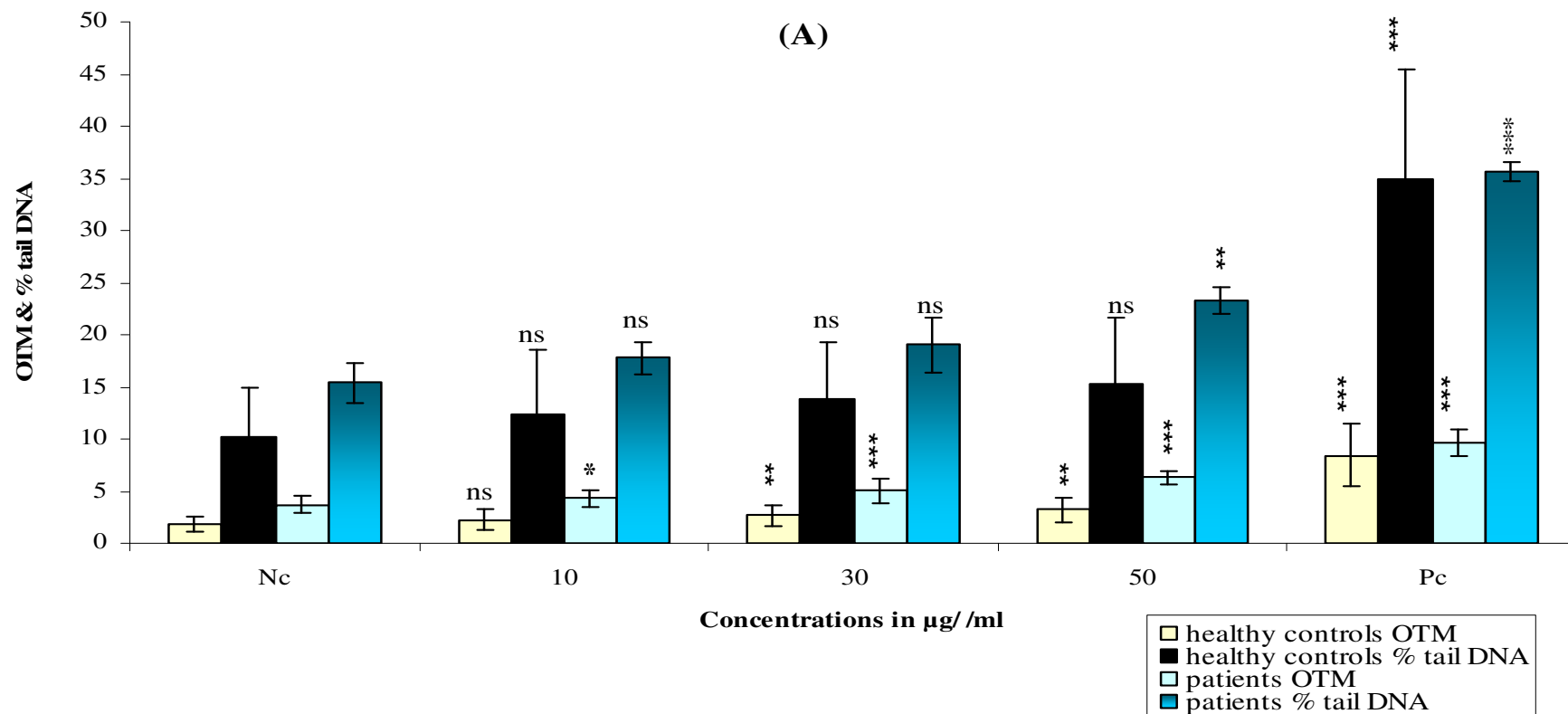


Figure 5.1A Histogram showing the means of Olive tail moment and % tail DNA in peripheral blood lymphocytes of the healthy controls compared to the DNA damage in the respiratory disease group of patients in the Comet assay after treatment with different TiO₂ concentrations

(10, 30 and 50 $\mu\text{g/ml}$), as well as the negative control of untreated lymphocytes (Nc) and the positive control of 80 μM (2.72 $\mu\text{g/ml}$) H_2O_2 (Pc) for 30 minutes. Bars indicate standard errors. Not significant: ns, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

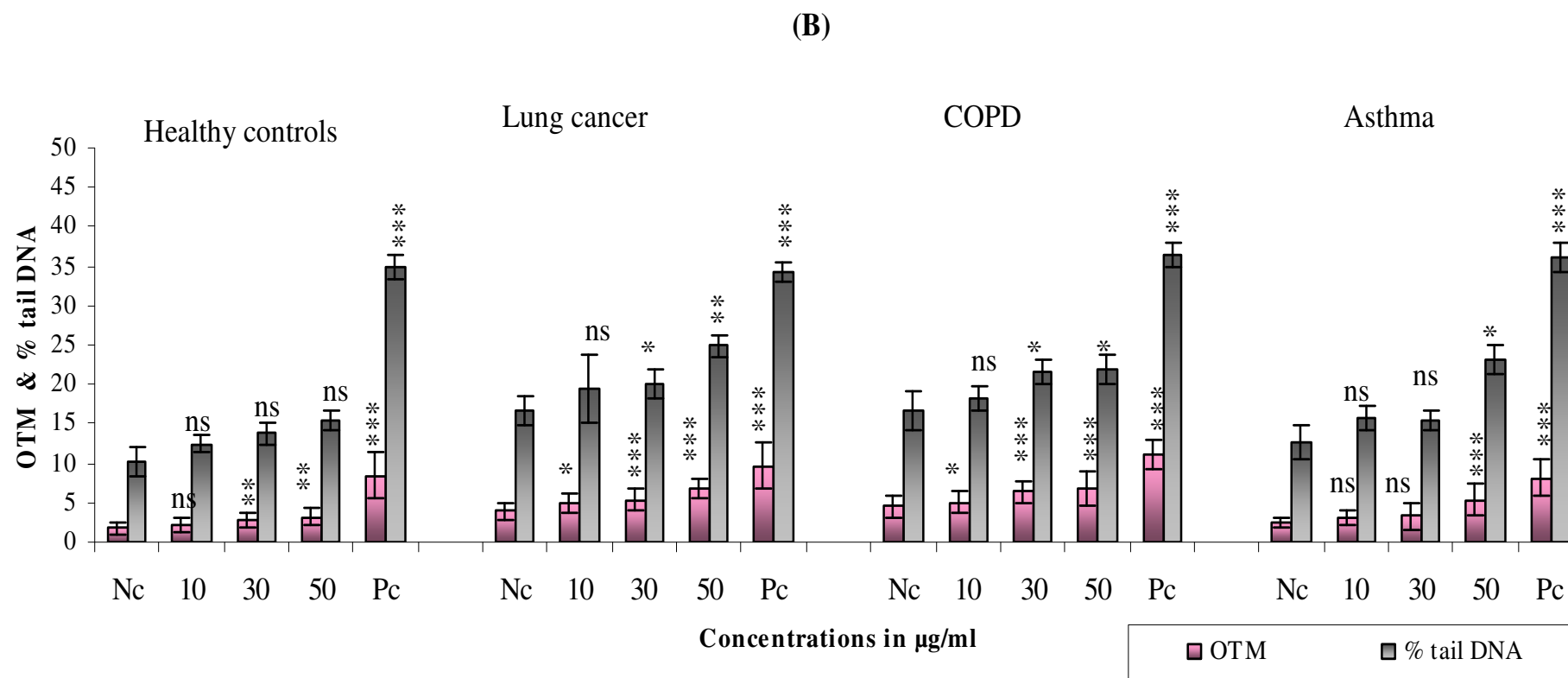


Figure 5.1B Histogram showing the means of Olive tail moment and % tail DNA in lymphocytes of healthy controls, lung cancer, COPD and asthma patient groups in the Comet assay after treatment with different TiO_2 concentrations (10, 30 and 50 $\mu\text{g/ml}$), as well as the negative control of untreated lymphocytes (Nc) and the positive control of 80 μM (2.72 $\mu\text{g/ml}$) H_2O_2 (Pc) for 30 minutes. Bars indicate standard errors. Not significant: ns, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

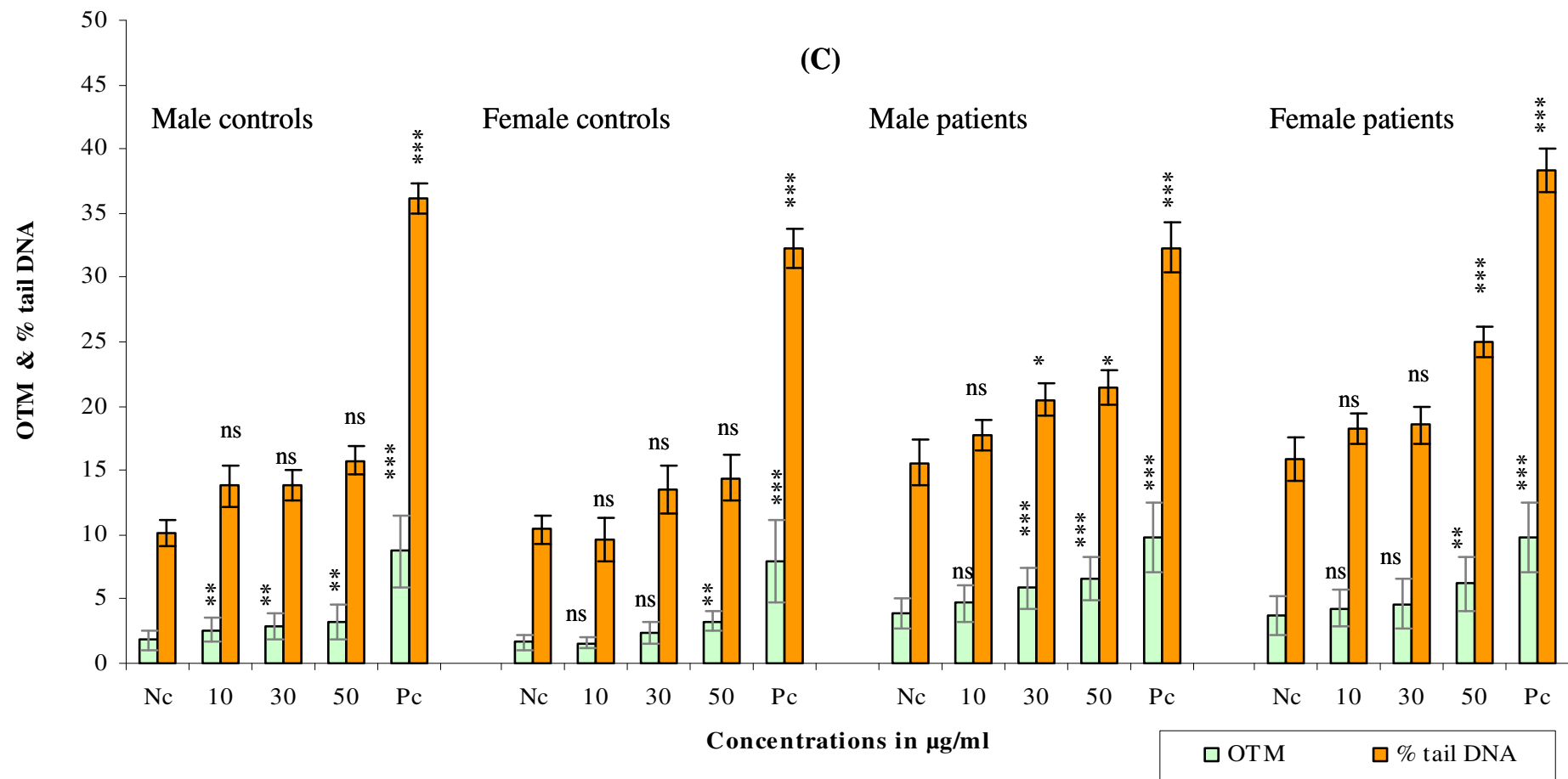


Figure 5.1C Histogram showing the means of Olive tail moment and % tail DNA in the peripheral blood lymphocytes of male healthy controls, female healthy controls, male patients and female patients groups in the Comet assay after treatment with different TiO₂ concentrations (10, 30 and 50 µg/ml), as well as the negative control of untreated lymphocytes (Nc) and the positive control of 80 µM (2.72 µg/ml) H₂O₂ (Pc) for 30 minutes. Bars indicate standard errors. Not significant: ns, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

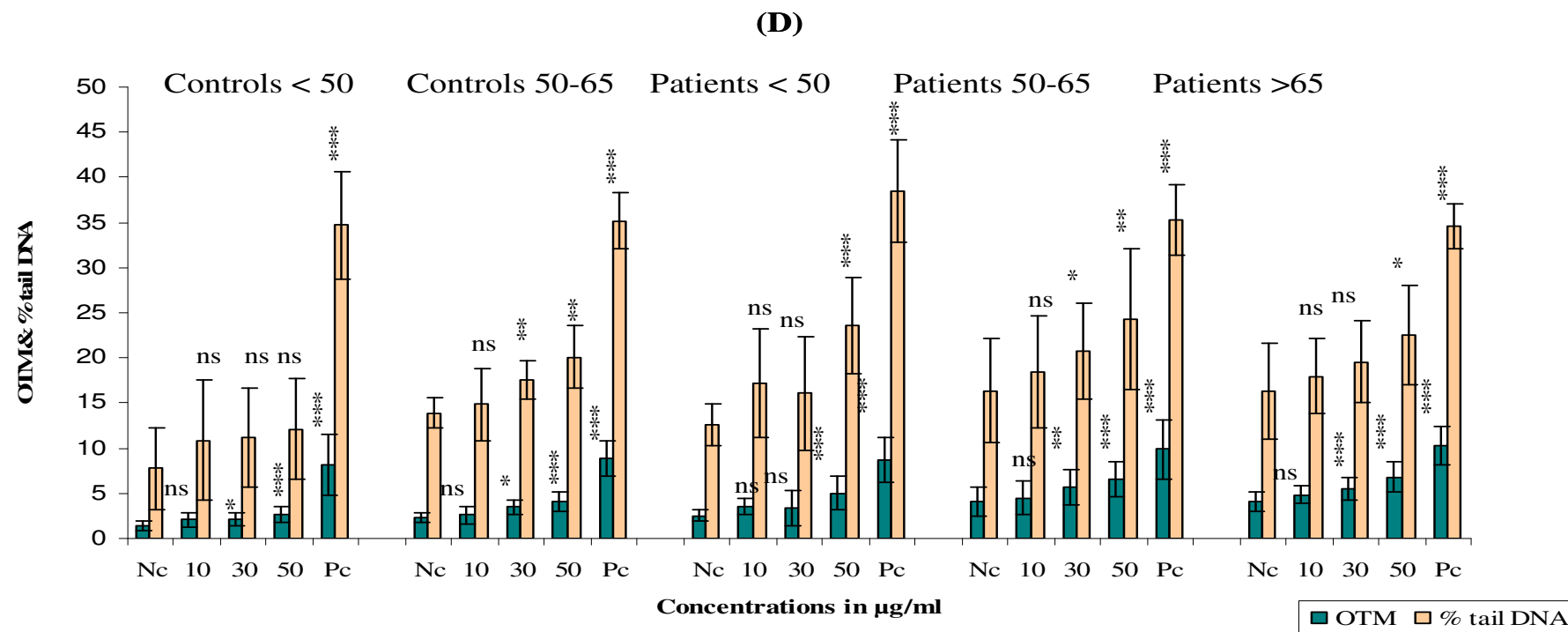


Figure 5.1D Histogram showing the means of Olive tail moment and % tail DNA in lymphocytes of healthy controls aged < 50 and 50-65, patients aged < 50, 50-65 and > 65 years old in the Comet assay after treatment with different TiO₂ concentrations (10, 30 and 50 µg/ml), as well as the negative control of untreated lymphocytes (Nc) and the positive control of 80 µM (2.72 µg/ml) H₂O₂ (Pc) for 30 minutes. Bars indicate standard errors. Not significant: ns, **p* < 0.05, ** *p* < 0.01 and ****p* < 0.001

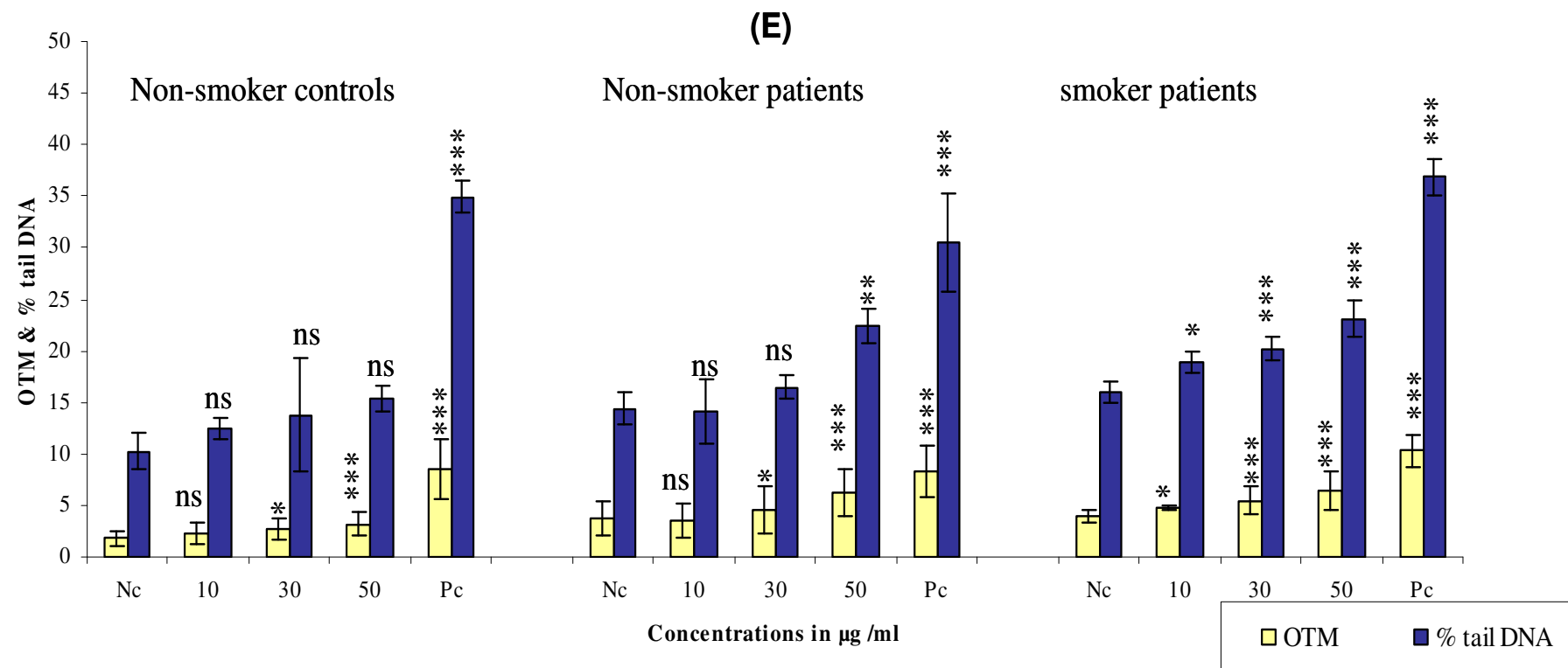


Figure 5.1E Histogram showing the means of Olive tail moment and % tail DNA in lymphocytes of non smoker healthy controls, non smoker and smoker patient groups in the Comet assay after treatment with different TiO_2 concentrations (10, 30 and 50 $\mu\text{g/ml}$), as well as the negative control of untreated lymphocytes (Nc) and the positive control of 80 μM (2.72 $\mu\text{g/ml}$) H_2O_2 (Pc) for 30 minutes. Bars indicate standard errors. Not significant: ns, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

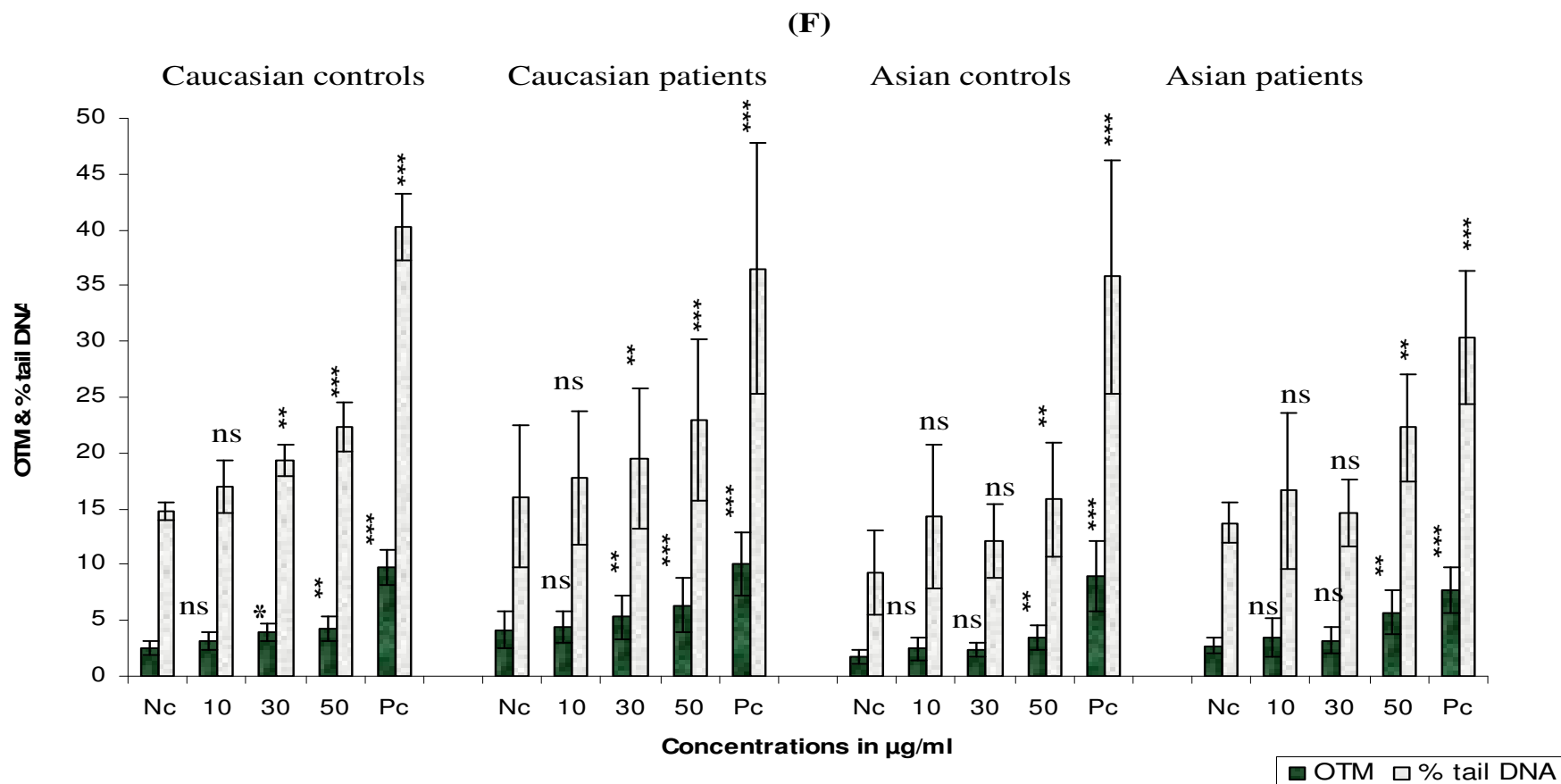


Figure 5.1F Histogram showing the means of Olive tail moment and % tail DNA in lymphocytes of Caucasian patients and controls, and Asian patients and control groups in the Comet assay after treatment with different TiO_2 concentrations (10, 30 and 50 $\mu\text{g/ml}$), as well as the negative control of untreated lymphocytes (Nc) and the positive control of 80 μM (2.72 $\mu\text{g/ml}$) H_2O_2 (Pc) for 30 minutes. Bars indicate standard errors. Not significant: ns, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

Table 5.3: Means \pm SD of the Olive tail moment (OTM) in lymphocytes of healthy controls and respiratory disease patients divided according to confounding factor. In each group the means of OTM of treated lymphocytes was compared with the means of OTM of the negative control of untreated lymphocytes (Nc) with ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and ^{n.s} not significant. (■ not significant ● $p < 0.05$, ◆ $p < 0.01$, ♣ $p < 0.001$) when patients and their confounders were compared with corresponding control samples. The positive control (Pc) was 80 μ M (2.72 μ g/ml) H₂O₂ was included.

Group type and confounder		Nc	TiO ₂ concentrations in μ g/ml			Pc
			10	30	50	
Control and patients groups	Healthy controls	1.8 \pm 0.7	2.3 \pm 1.0 ^{n.s}	2.7 \pm 1.0**	3.2 \pm 1.2**	8.5 \pm 3.0***
	Patient group	3.7 \pm 1.5◆	4.3 \pm 1.6*◆	5.0 \pm 2.0***♣	6.2 \pm 2.2***♣	9.3 \pm 3.2***
	Lung cancer	3.9 \pm 1.2◆	4.8 \pm 1.2*♣	5.3 \pm 1.4***♣	6.7 \pm 1.2***♣	9.7 \pm 2.9***
	COPD	4.6 \pm 1.4♣	5.0 \pm 1.4 ^{n.s} ♣	6.4 \pm 1.4***♣	6.8 \pm 2.2***♣	11.1 \pm 1.2***
	Asthma	2.6 \pm 0.6■	3.2 \pm 0.9 ^{n.s} ■	3.4 \pm 1.7 ^{n.s} ■	5.3 \pm 2.0***●	8.2 \pm 2.3***
Gender	Male controls	1.8 \pm 0.8	2.6 \pm 1.0**	2.9 \pm 1.0**	3.2 \pm 1.3**	8.7 \pm 2.7***
	Female controls	1.7 \pm 0.6	1.6 \pm 0.5 ^{n.s}	2.4 \pm 0.8 ^{n.s}	3.3 \pm 0.8**	8.0 \pm 3.3***
	Male patients	3.9 \pm 1.2◆	4.7 \pm 1.4 ^{n.s} ◆	5.9 \pm 1.6***♣	6.6 \pm 1.7***♣	9.8 \pm 2.8***
	Female patients	3.8 \pm 1.5◆	4.3 \pm 1.4 ^{n.s} ♣	4.61 \pm 1.90 ^{n.s} ●	6.2 \pm 2.1**◆	9.85 \pm 2.69***
Age	Controls < 50	1.4 \pm 0.5	2.0 \pm 0.9 ^{n.s}	2.2 \pm 0.7*	2.6 \pm 0.9***	8.2 \pm 3.4***
	Controls 50-65	2.4 \pm 0.6	2.6 \pm 1.0 ^{n.s}	3.5 \pm 0.8*	4.1 \pm 1.0***	8.9 \pm 1.9***
	Patients < 50	2.6 \pm 0.6■	3.6 \pm 0.9 ^{n.s} ■	3.4 \pm 1.9 ^{n.s} ●	5.0 \pm 1.9***◆	8.6 \pm 2.5***
	Patients 50-65	4.0 \pm 1.6◆	4.5 \pm 1.8 ^{n.s} ◆	5.7 \pm 2.0***♣	6.5 \pm 1.9***◆	9.8 \pm 3.2***
	Patients >65	4.1 \pm 1.1♣	4.8 \pm 1.0 ^{n.s} ♣	5.4 \pm 1.2***♣	6.8 \pm 1.7***♣	10.2 \pm 2.1***
Smoking	Non-smoking Controls	1.8 \pm 0.7	2.3 \pm 1.0 ^{n.s}	2.7 \pm 1.0*	3.2 \pm 1.2***	8.5 \pm 3.0***
	Non-smoking patients	3.7 \pm 1.7◆	3.5 \pm 1.7 ^{n.s} ●	4.5 \pm 2.3*◆	6.3 \pm 2.3***♣	8.3 \pm 2.6***
	Smoking patients	3.9 \pm 1.3◆	4.7 \pm 1.2*♣	5.4 \pm 1.6***♣	6.4 \pm 1.8***♣	10.3 \pm 2.6***
Ethnicity	Caucasian controls	2.5 \pm 0.6■	3.2 \pm 0.8 ^{n.s} ■	4.0 \pm 0.8*■	4.3 \pm 1.1**■	9.7 \pm 1.6***
	Caucasian patients	4.2 \pm 1.6●	4.5 \pm 1.4 ^{n.s} ■	5.3 \pm 1.9**◆	6.3 \pm 2.5***■	10.1 \pm 2.8***
	Asian controls	1.7 \pm 0.6	2.5 \pm 1.0 ^{n.s}	2.4 \pm 0.6 ^{n.s}	3.4 \pm 1.1**	8.9 \pm 3.1***
	Asian patients	2.8 \pm 0.7	3.5 \pm 1.7 ^{n.s}	3.2 \pm 1.1 ^{n.s}	5.7 \pm 2.0**	7.7 \pm 2.1***

Table 5.4: Means \pm SD of % tail DNA in lymphocytes of healthy controls and respiratory diseases patients divided according to confounding factor. In each group the means of % tail DNA of treated lymphocytes was compared with the means of the negative controls % tail DNA of untreated lymphocytes (Nc) with ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and ^{ns} not significant. (■ not significant ● $p < 0.05$, ◆ $p < 0.01$, ▲ $p < 0.001$) when patients and their confounders were compared with corresponding control samples. The positive control (Pc) of 80 μ M (2.72 μ g/ml) H₂O₂ was included.

Group type and confounder		Nc	TiO ₂ concentrations in μ g/ml			Pc
			10	30	50	
Control and patients groups	Healthy controls	10.2 \pm 4.7	12.4 \pm 6.1 ^{ns}	13.8 \pm 5.5 ^{ns}	15.3 \pm 6.3 ^{ns}	34.9 \pm 10.6***
	Patient group	15.4 \pm 5.3◆	17.7 \pm 5.4 ^{ns} ◆	19.0 \pm 5.5 ^{ns} ▲	23.3 \pm 6.5***▲	35.6 \pm 9.3***
	Lung cancer	16.8 \pm 5.8◆	19.4 \pm 4.4 ^{ns} ◆	20.1 \pm 4.9 *▲	24.9 \pm 6.4***▲	34.4 \pm 9.3***
	COPD	16.7 \pm 5.5◆	18.2 \pm 5.6 ^{ns} ◆	21.6 \pm 4.5*▲	21.9 \pm 6.8*▲	36.5 \pm 9.6***
	Asthma	12.7 \pm 2.0■	15.6 \pm 5.5 ^{ns} ■	15.4 \pm 5.3 ^{ns} ■	23.1 \pm 5.8*▲	36.0 \pm 8.8***
Gender	Male controls	10.1 \pm 5.0	13.8 \pm 6.6 ^{ns}	13.8 \pm 5.3 ^{ns}	15.7 \pm 3.8 ^{ns}	36.2 \pm 12.5***
	Female controls	10.4 \pm 4.1	9.69 \pm 3.7 ^{ns}	13.6 \pm 5.6 ^{ns}	14.4 \pm 5.8 ^{ns}	32.3 \pm 8.8***
	Male patients	15.9 \pm 5.7◆	17.7 \pm 4.2 ^{ns} ■	20.5 \pm 5.2*◆	21.5 \pm 7.1*●	32.3 \pm 9.2***
	Female patients	15.9 \pm 4.8■	18.3 \pm 6.2 ^{ns} ◆	18.5 \pm 5.5 ^{ns} ■	25.1 \pm 6.2***▲	38.4 \pm 7.7***
Age	Controls < 50	7.8 \pm 4.5	10.8 \pm 6.6 ^{ns}	11.2 \pm 5.5 ^{ns}	12.1 \pm 6.7 ^{ns}	34.7 \pm 11.9***
	Controls 50-65	13.9 \pm 2.4◆	14.8 \pm 6.0 ^{ns} ●	17.5 \pm 6.3***◆	20.0 \pm 3.5***■	35.2 \pm 8.1***
	Patients < 50	12.6 \pm 1.7◆	17.2 \pm 4.0 ^{ns} ◆	16.1 \pm 2.2 ^{ns} ◆	23.6 \pm 5.4***■	38.5 \pm 9.7***
	Patients 50-65	16.4 \pm 5.8▲	18.4 \pm 6.2 ▲ ^{ns}	20.8 \pm 5.4*▲	24.2 \pm 3.5***■	35.3 \pm 8.1***
	Patients >65	16.3 \pm 5.3▲	17.9 \pm 4.2 ▲ ^{ns}	19.6 \pm 4.6 ▲ ^{ns}	22.5 \pm 7.8■	34.6 \pm 9.8***
Smoking	Non-smoking Controls	10.2 \pm 4.7	12.4 \pm 6.1 ^{ns}	13.7 \pm 5.5 ^{ns}	15.3 \pm 6.3 ^{ns}	34.9 \pm 10.6***
	Non-smoking patients	14.4 \pm 3.5◆	14.1 \pm 3.1 ^{ns} ◆	16.5 \pm 5.2 ^{ns} ◆	22.4 \pm 6.6***▲	30.5 \pm 6.8***
	Smoking patients	15.9 \pm 5.1◆	18.9 \pm 5.0*▲	20.2 \pm 5.1***▲	23.1 \pm 9.6***▲	36.8 \pm 9.3***
Ethnicity	Caucasian controls	14.8 \pm 0.7■	17.0 \pm 2.3 ^{ns} ■	19.3 \pm 1.4***■	22.4 \pm 2.2***■	40.2 \pm 3.0***
	Caucasian patients	16.1 \pm 6.36■	17.7 \pm 6.0 ^{ns} ■	19.5 \pm 6.3***●	22.9 \pm 7.2***■	36.6 \pm 11.2***
	Asian controls	9.3 \pm 3.8	14.3 \pm 6.4 ^{ns}	12.2 \pm 3.3 ^{ns}	15.8 \pm 5.1**	35.8 \pm 10.4***
	Asian patients	13.8 \pm 1.8	16.6 \pm 7.0 ^{ns}	14.6 \pm 3.0 ^{ns}	22.3 \pm 4.8**	30.3 \pm 6.0***

5.3.2 CBMN assay in lymphocytes from respiratory disease patients and controls

A total of 46 individuals from four groups were screened for MN, consisting of 13 lung cancer, 11 COPD, 8 asthma and 15 controls. These samples were further divided to test for the confounding factors (age, gender and smoking habit), to determine whether these factors could influence our findings. All former smokers and those who never smoked were considered as non-smokers to increase the statistical power of detection of any assumed association of MN with smoking. MMC was included as a system control and the responses were as anticipated indicating that the system and the whole experiment had worked.

Each sample was treated with two TiO₂ concentrations (5 and 10µg/ml), a positive control (0.4µ MMC) and a negative control of untreated cells (figure 5.2 A-F and table 5.5). The nuclear division index (NDI) was evaluated with the Chi square test, and the number of MN in 1000 binucleated cells was compared between the untreated and treated for each sample using the graph pad 2x2 contingency (Fisher exact test) for the P value. An extra comparison was carried out between the patients' samples and the healthy control untreated sample. To assess the effect of other factors such as co-morbidity on MN frequency, patients and controls were further divided to subgroups according to their gender, age, smoking habit and ethnicity as shown in figure (5.2 C-F).

5.3.2.1 Patients and controls

Although, figure 5.2 A-F and table 5.5 have shown that the number of micronuclei increases, the difference were not statistically significant, except when the MN in patient samples compared to the MN of untreated control. This showed a significant increase in MN induction in all patients groups including for their confounding factors.

In all groups as indicated in table 5.5.1 the nuclear division index (NDI) and the percentage bi-nucleated cells (% BiNC) which are indicators of the cytostatic state were within normal limits. MMC the positive control behaved as anticipated signifying that the system and the whole experiment were working satisfactory. In all TiO₂ treated samples there was an increase in NPBs and to lesser extent buds which were not significant.

Figure 5.2A, shows a clear difference in MN numbers between the lung disease patients and the healthy controls which was not significant except when comparing patients' MN to those of untreated healthy controls MN the patients' induced MN was significant ($p < 0.05$) (figure 5.2A and table 5.5.1).

5.3.2.2 Analysis of confounding factors:

5.3.2.2.1 Lung cancer, COPD, asthma and healthy controls

Figure 5.2 B and table 5.5 the MN frequency distribution in controls and lung cancer, COPD and asthma patient groups, was displayed in figure 5.2B, which demonstrated an increasing pattern of MN induction in each of the above mentioned groups; however it was not statistically significant. Thereafter, we compared MN induced by lung cancer, COPD and asthma patients to the MN of the untreated control of healthy individuals and a significant difference was reported for all patient groups, at $p < 0.01$ for lung cancer and COPD and $p < 0.05$ for asthma.

5.3.2.2.2 Gender

According to gender, male and female patients showed higher MN which was statistically significant compared to the untreated control of the healthy persons group at $p < 0.01$ for both the male and female lymphocytes response to TiO₂ concentration of

TiO₂ 10 µg/ml. However, only male patients showed a response to (Nc) and 5 µg/ml at $p < 0.05$ (figure 5.2C and table 5.5.2).

5.3.2.2.3 Age

In relation to age, the two older patient groups induced significantly higher MN compared to the untreated control of healthy individuals ($p < 0.01$) and was not significant compared to their negative controls (figure 5.2D and table 5.5.3).

5.3.2.2.4 Smoking

The smoking and non smoking patients showed statistically significant increases in MN number when compared to the control of the healthy individuals for both TiO₂ concentrations. However, the induced MN number of the smoking and non smoking patients was not significant compared to its counterpart untreated control (figure 5.2E and table 5.5.4).

5.3.2.2.5 Ethnicity

Ethnicity, as a confounder was tested on 30 Caucasian and 6 Asian patients and 10 Caucasian and 5 Asian controls. Caucasian patients showed the largest increase in the MN number compared to the other groups (figure 5.2F and table 5.5.5). Results were not significant unless compared to the untreated control groups. The higher significance obtained was for the Asian patients for the number of MN in 1000 binucleated cells.

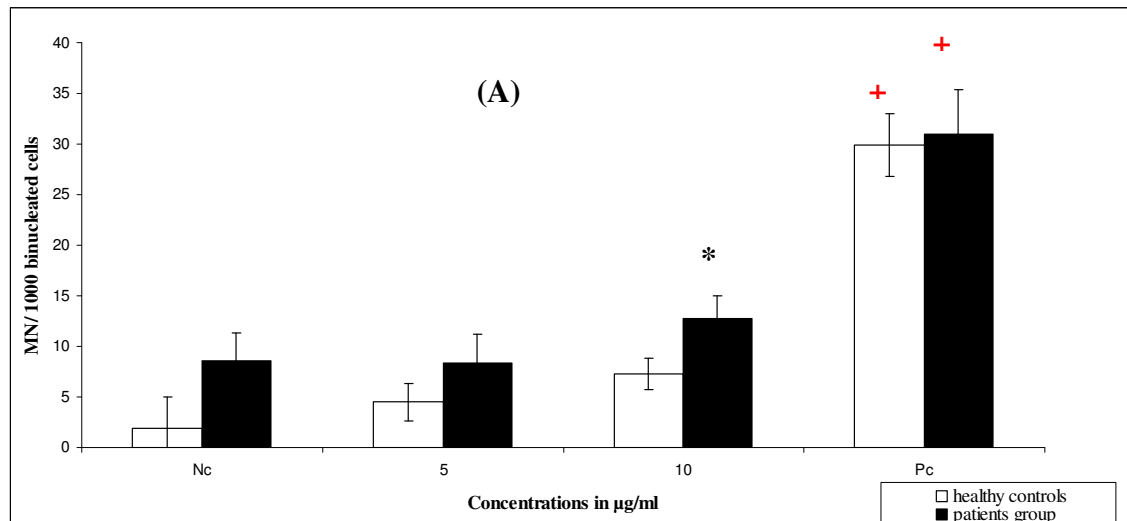


Figure 5.2A Histogram showing the means of micronuclei (MN) per 1000 binucleated cells of healthy controls and respiratory disease group of patients after treatment of blood cultures with 2 different TiO_2 concentrations (5 and $10\mu\text{g/ml}$) as well as the negative control of untreated blood cultures (Nc) and the positive control of $0.4\mu\text{M}$ MMC (Pc) in the CBMN assay. *denotes $p < 0.05$ and ** $p < 0.01$ when the patients group are compared with untreated lymphocytes of the controls group. +denotes highly significant in both situations when compared with the untreated control of their own specific group, as well as with the untreated lymphocytes of healthy controls. The bars indicate standard errors.

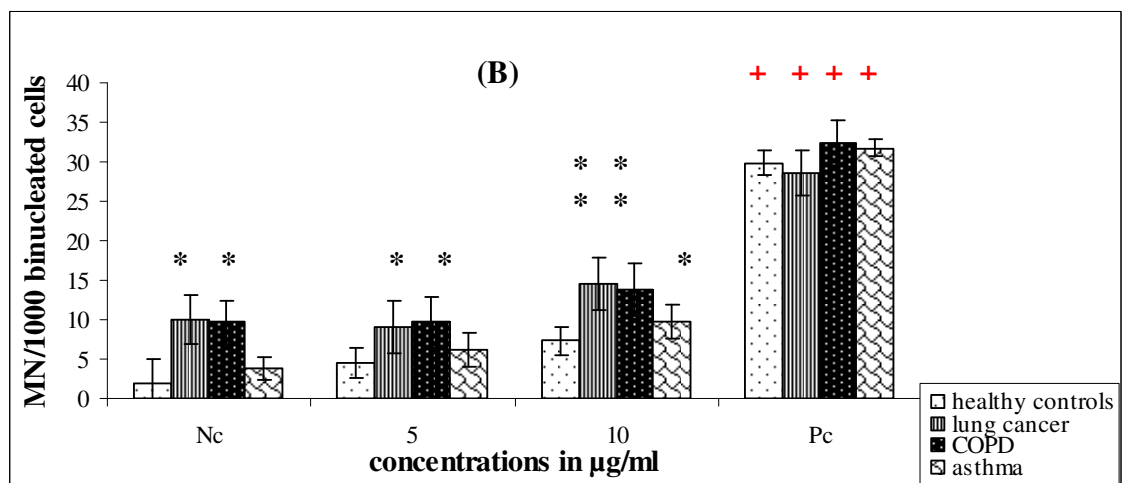


Figure 5.2B Histogram showing the means of micronuclei (MN) per 1000 binucleated cells of healthy controls, lung cancer, COPD and asthma patients after treatment of blood cultures with 2 different TiO_2 concentrations (5 and $10\mu\text{g/ml}$) as well as the negative control of untreated blood cultures (Nc) and the positive control of $0.4\mu\text{M}$ MMC (Pc) in the CBMN assay. * denotes $p < 0.05$ and ** $p < 0.01$ when the patient groups are compared with untreated lymphocytes of the controls. +denotes highly significant in both situations when compared with the untreated control of their own specific group, as well as with the untreated lymphocytes of healthy controls. The bars indicate standard errors.

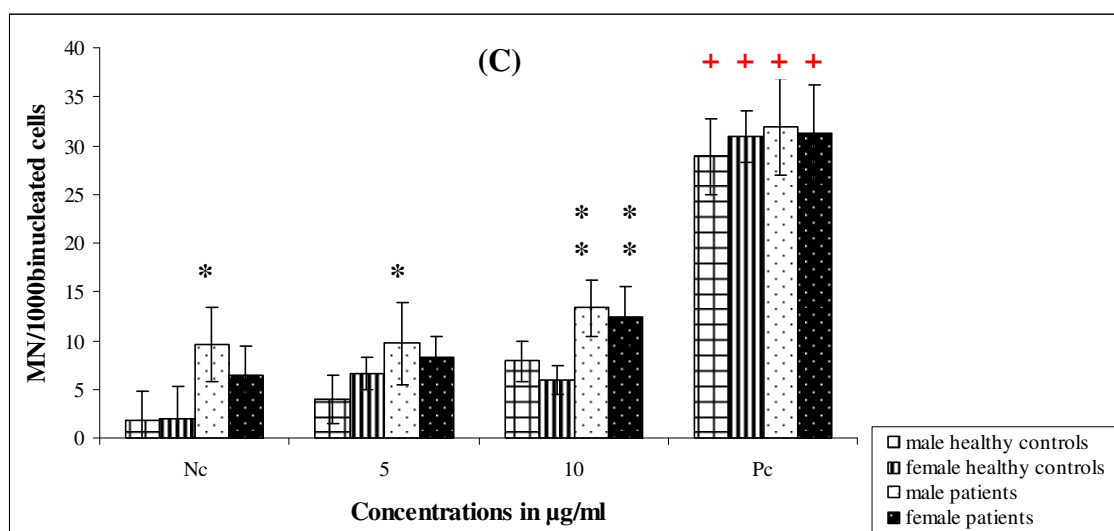


Figure 5.2C Histogram showing the means of micronuclei (MN) per 1000 binucleated cells of male healthy controls, female healthy controls, male respiratory disease patients and female respiratory disease patients after treatment of blood cultures with 2 different TiO_2 concentrations (5 and $10\mu\text{g/ml}$) as well as the negative control of untreated blood cultures (Nc) and the positive control of $0.4\mu\text{M}$ MMC (Pc) in the CBMN assay. * denotes $p < 0.05$ and ** $p < 0.01$ when the patient groups are compared with untreated lymphocytes of the controls. + denotes highly significant in both situations when compared with the untreated controls of their own specific group, as well as with the untreated lymphocytes of healthy controls. The bars indicate standard errors.

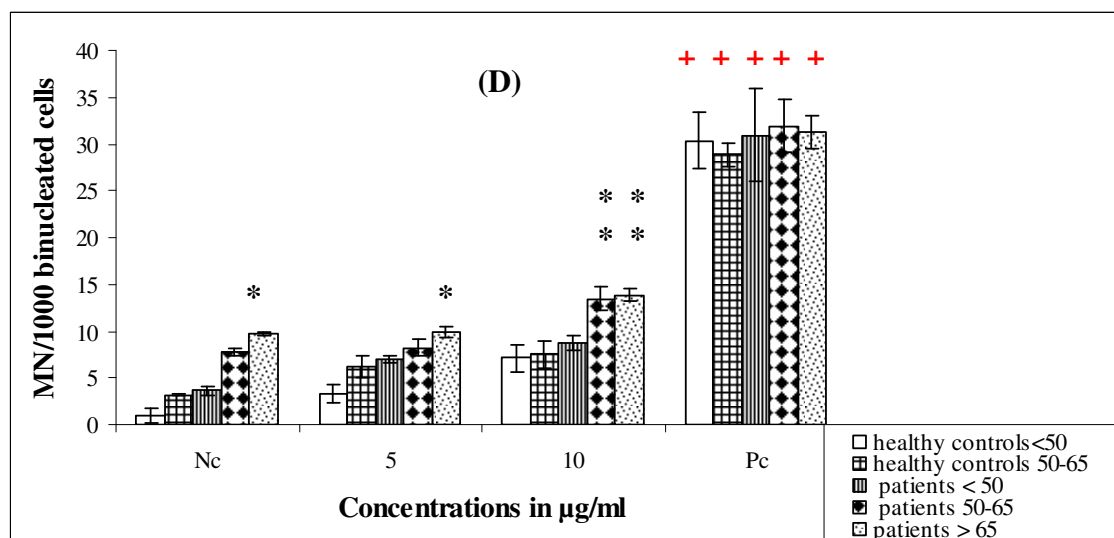


Figure 5.2D Histogram showing the means of micronuclei (MN) per 1000 binucleated cells of age < 50 healthy controls, age 50-65 healthy controls, age < 50 patients, age 50-65 patients and patients aged > 65 after treatment of blood cultures with 2 different TiO_2 concentrations (5 and $10\mu\text{g/ml}$) as well as the negative control of untreated blood cultures (Nc) and the positive control of $0.4\mu\text{M}$ MMC (Pc) in the CBMN assay. * denotes $p < 0.05$ and ** $p < 0.01$ when the patient groups are compared with untreated lymphocytes of the controls. + denotes highly significant in both situations when compared with the untreated control of their own specific group as well as with the untreated lymphocytes of healthy controls. The bars indicate standard errors.

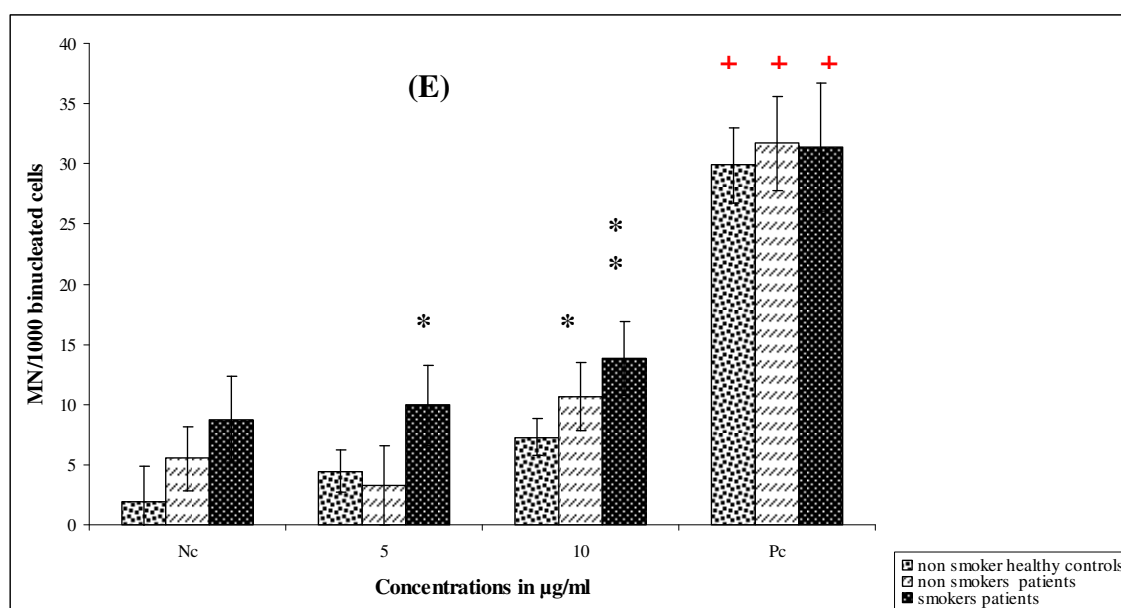


Figure 5.2E Histogram showing the means of micronuclei (MN) per 1000 binucleated cells of non smoker healthy controls, non smoker patients and smoker patients after treatment of blood cultures with 2 different TiO_2 concentrations (5 and $10\mu\text{g/ml}$) as well as the negative control of untreated blood cultures (Nc) and the positive control of $0.4\mu\text{M}$ MMC (Pc) in the CBMN assay. *denotes $p < 0.05$ and ** $p < 0.01$ when the patient groups are compared with untreated lymphocytes of the controls. +denotes highly significant in both situations when compared with the untreated control of their own specific group as well as with the untreated lymphocytes of healthy controls. The bars indicate standard errors.

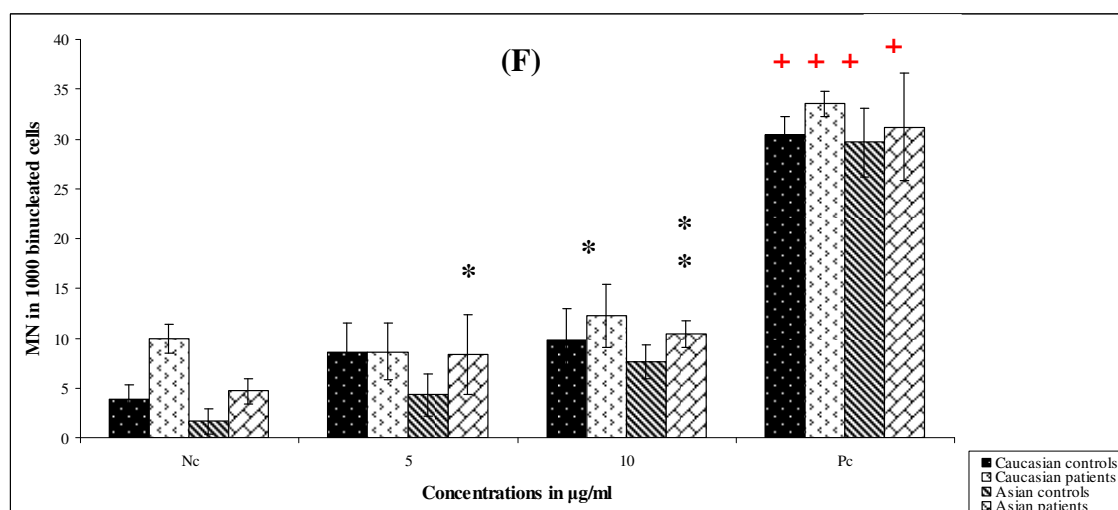


Figure 5.2F Histogram showing the means of micronuclei (MN) per 1000 binucleated cells of Caucasian controls, Caucasian patients, Asian controls and Asian patients after treatment of blood cultures with 2 different TiO_2 concentrations (5 and $10\mu\text{g/ml}$) as well as the negative control of untreated blood cultures (Nc) and the positive control of $0.4\mu\text{M}$ MMC (Pc) in the CBMN assay. *denotes $p < 0.05$ and ** $p < 0.01$ when the patient groups are compared with untreated lymphocytes of the controls. +denotes highly significant in both situations when compared with the untreated control of their own specific group as well as with the untreated lymphocytes of healthy controls. The bars indicate standard errors.

Table 5.5.1: Health condition as a factor in the CBMN. Means \pm SD of different CBMN assay parameters of respiratory disease patients (lung cancer, COPD, asthma) and healthy controls in blood cultures treated with TiO₂ (5 and 10 μ g/ml) as well as a negative control of untreated blood cultures (Nc) and a positive control (Pc) of 0.4 μ M of mitomycin C (MMC). Symbols indicate level of significance when comparing each group to healthy individuals' untreated controls. * implies $P < 0.05$, ** $P < 0.01$ and ⁺ denotes highly significant when compared each group to its own untreated control as well as to corresponding healthy individuals untreated control.

Group type and confounder		Nc	TiO ₂ concentrations µg/ml		Pc
			5	10	
Control and patients groups	Controls group				
	NDI	1.88 ± 0.03	1.84 ± 0.02	1.73 ± 0.02	1.66 ± 0.03
	%BiNC	58.04 ± 4.42	56.35± 2.34	58.34 ± 3.45	46 ± 2.44
	BiMN	1.87 ± 1.63	4.47 ± 2.39	7.27 ± 1.69	29.87 ± 4.15 ⁺
	Mono MN	1.93 ± 2.24	1.80 ± 1.51	3.67 ± 1.51	5.2 ± 2.56
	NPBs	0.33 ± 0.47	4.40 ± 2.95	4.80 ± 2.45	0.53 ± 0.81
	Nuclear buds	0.67 ± 0.79	2.13 ± 1.64	2.13 ± 1.5	0.72 ± 0.47
	Patients group				
	NDI	1.68 ± 0.03	1.54 ± 0.02	1.67 ± 0.08	1.52 ± 0.05
	%BiNC	48.50 ± 1.68	46.40 ± 3.45	49.60 ± 2.87	43.45 ± 3.75
	BiMN	8.54 1.40±	8.29 ± 1.55	11.03 ± 1.70 [*]	30.90 ± 12.21 ⁺
	Mono MN	2.04 ± 0.48	3.06 ± 0.28	3.95 ± 1.33	1.21 ± 0.13
	NPBs	5.32 ± 0.06	5.39 ± 0.45	4.79 ± 6.65	5.42 ± 0.24
	Nuclear buds	2.58 ± 0.17	1.88 ± 0.73	1.96 ± 0.98	1.42 ± 0.27
	Lung cancer				
	NDI	1.65 ± 0.06	1.66 ± 0.03	1.65 ± 0.03	1.55 ± 5.2
	%BiNC	54.70 ± 2.34	52.50 ± 4.56	56.34 ± 3.42	37.35 ± 2.34
	BiMN	9.98 ± 2.40 [*]	9.71 ± 3.35 [*]	14.50 ± 3.01 ^{**}	28.58 ± 4.39 ⁺
	Mono MN	2.58 ± 2.60	3.33 ± 1.75	5.83 ± 2.11	1.08 ± 1.66
	NPBs	1.08 ± 1.11	5.67 ± 1.89	5.33 ± 2.53	1.50 ± 1.85
	Nuclear buds	0.83 ± 0.99	2.58 ± 2.26	1.33 ± 1.17	1.42 ± 1.04
	COPD				
	NDI	1.63 ± 0.02	1.59 ± 0.03	1.62 ± 0.04	1.38 ± 0.06
	%BiNC	49.60 ± 2.35	52.25 ± 2.33	56 ± 3.44	46.34 ± 1.36
	BiMN	9.67 ± 2.61 [*]	9.67 ± 3.25 [*]	13.83 ± 1.95 ^{**}	32.33 ± 4.37 ⁺
	Mono MN	1.42 ± 1.38	3.17 ± 3.02	2.92 ± 2.66	1.17± 1.28
	NPBs	1.3 ± 1.43	5.75 ± 3.29	6.08 ± 2.69	2.58 ± 1.89
	Nuclear buds	1.17 ± 1.14	2.17 ± 2.94	3.42 ± 1.44	1.08 ± 1.11
	Asthma				
	NDI	1.86 ± 0.08	1.82 ± 0.02	1.67 ± 0.06	1.42 ± 0.06
%BiNC	45.55 ± 2.06	65.23 ± 3.44	61.44 ± 2.44	45.2 ± 5.65	
BiMN	3.75 ± 1.48	6.13 ± 2.15	9.73 ± 1.54 [*]	31.75 ± 4.92 ⁺	
Mono MN	2.13 ± 1.69	2.6 ± 1.87	3.1 ± 2.15	1.38 ± 1.41	
NPBs	1.375 ± 1.5	4.75 ± 2.38	3.88 ± 3.22	2.63 ± 1.49	
Nuclear buds	0.75 ± 1.09	0.88 ± 1.05	1.13 ± 1.05	1.75 ± 0.97	

Footnote:

NDI: indicates nuclear division index

% Bi NC: indicates percentage binucleated cells in 1000 scored cells

BiMN: indicates number of micronuclei in 1000 binucleated cells

Mono MN: indicates micronuclei in mononucleated counted in 1000 cells.

NPBs: indicates number of nucleoplasmic bridges in 1000 cell

Table 5.5.2: Gender as a confounding factor in the CBMN assay. Means \pm SD of different CBMN assay parameters of respiratory disease patients (lung cancer, COPD, asthma) and healthy controls in 1 blood cultures treated with TiO₂ (5 and 10 μ g/ml) as well as a negative control of untreated blood cultures (Nc) and a positive control (Pc) of 0.4 μ M of mitomycin C (MMC). Symbols indicate level of significance when comparing each group to healthy individuals' untreated controls. * $P < 0.05$, ** $P < 0.01$ and ⁺denotes highly significant when compared each group to its own untreated control as well as to corresponding healthy individuals untreated control.

Group type and confounder		Nc	TiO ₂ concentrations μ g/ml		Pc
			5	10	
Gender	Healthy males				
	NDI	1. 88 \pm 0.02	1.88 \pm 0.04	1. 72 \pm 0.02	1.56 \pm 0.04
	%BiNC	54.45 \pm 3.22	52.28 \pm 4.66	50.44 \pm 6.33	38.44 \pm 1.01
	BiMN	1.8 \pm 1.6	6.4 \pm 0.34	7.9 \pm 1.37	28.87 \pm 2.81 ⁺
	Mono MN	1.93 \pm 2.24	1.80 \pm 1.51	3.67 \pm 1.58	5.20 \pm 2.56
	NPBs	1.8 \pm 0.3	2.1 \pm 1.65	5.21 \pm 2.27	0.87 \pm 0.8
	Nuclear buds	0.78 \pm 0.7	1.12 \pm 0.97	2.73 \pm 1.35	0.81 \pm 0.5
	Healthy females				
	NDI	1.88 \pm 0.06	1.80 \pm 0.02	1.74 \pm 0.04	1.76 \pm 0.02
	%BiNC	64.12 \pm 1.24	55.45 \pm 2.02	56.46 \pm 0.04	48.26 \pm 1.22
	BiMN	2 \pm 1.67	6.60 \pm 2.42	6.00 \pm 1.55	30.01 \pm 5.51 ⁺
	Mono MN	2.14 \pm 2.24	3.27 \pm 2.49	4.09 \pm 2.79	1.23 \pm 1.56
	NPBs	0.60 \pm 0.49	4.40 \pm 1.36	4,22 \pm 2.61	0. 0 \pm 0
	Nuclear buds	0.80 \pm 0.6	1.60 \pm 1.85	1.10 \pm 0.73	0.4 \pm 0.49
	Male patients				
	NDI	1.60 \pm 0.04	1.56 \pm 0.02	1.62 \pm 0.39	1. 70 \pm 0.02
	%BiNC	52.56 \pm 1.26	58.55 \pm 2.22	56.64 \pm 2.46	36.04 \pm 1.65
	BiMN	9.56 \pm 2.63*	9.69 \pm 4.16*	13.38 \pm 2.64 ^{**}	31.69 \pm 5.26 ⁺
	Mono MN	1.59 \pm 1.52	2.75 \pm 2.64	1.93 \pm 1.86	1.36 \pm 1.30
	NPBs	1.31 \pm 1.40	6.06 \pm 2.46	5.25 \pm 2.56	1.88 \pm 1.76
	Nuclear buds	1.11 \pm 0.88	2.00 \pm 1.84	2.50 \pm 2.09	1.31 \pm 1.10
	Female patients				
	NDI	1.76 \pm 0.02	1.52 \pm 0.02	1.72 \pm .0.02	1.34 \pm 0.04
	%BiNC	49.70 \pm 4.73	52.50 \pm 1.78	46.50 \pm 2.56	37.35 \pm 2.87
BiMN	6.50 \pm 3.34	8.25 \pm 2.22	12.44 \pm 3.62 ^{**}	30.91 \pm 4.30 ⁺	
Mono MN	2.00 \pm 2.00	1.75 \pm 1.55	3.60 \pm 1.74	5.3 \pm 2.28	
NPBs	1.29 \pm 1.19	5.25 \pm 2.54	5.25 \pm 3.21	2.50 \pm 1.90	
Nuclear buds	1.00 \pm 1.06	1.63 \pm 2.26	1.63 \pm 1.69	1.44 \pm 1.06	

Footnote:

NDI: indicates nuclear division index

% Bi NC: indicates percentage binucleated cells in 1000 scored cells

BiMN: indicates number of micronuclei in 1000 binucleated cells

Mono MN: indicates micronuclei in mononucleated counted in 1000 cells.

NPBs: indicates number of nucleoplasmic bridges in 1000 cells

Table 5.5.3: Age as a confounding factor in the CBMN assay. Means \pm SD of different CBMN assay parameters of respiratory disease patients (lung cancer, COPD, asthma) and healthy controls in blood cultures treated with TiO₂ (5 and 10 μ g/ml) as well as a negative control of untreated blood cultures (Nc) and a positive control (Pc) of 0.4 μ M of mitomycin C (MMC). Symbols indicate level of significance when comparing each group to healthy individuals' untreated controls. * $P < 0.05$, ** $P < 0.01$ and ⁺denotes highly significant when compared each group to its own untreated control as well as to corresponding healthy individuals untreated control.

Group type and confounder		TiO ₂ concentrations μ g/ml			Pc
		Nc	5	10	
Age	Control <50				
	NDI	1.88 \pm 0.03	1.74 \pm 0.02	1.76 \pm 0.04	1.38 \pm 0.03
	%BiNC	56.34 \pm 1.78	45.66 \pm 1.26	48.46 \pm 3.06	37.66 \pm 2.86
	BiMN	1.00 \pm 0.82	3.33 \pm 1.63	7.11 \pm 1.73	30.38 \pm 4.51 ⁺
	Mono MN	1.8 \pm 2.64	2.00 \pm 1.41	1.80 \pm 1.17	5.00 \pm 3.03
	NPBs	0.47 \pm 0.33	3.44 \pm 2.54	5.78 \pm 2.35	0.68 \pm 0.44
	Nuclear buds	0.68 \pm 0.44	1.89 \pm 1.10	2.22 \pm 1.40	0.68 \pm 0.56
	Control 50-65				
	NDI	1.84 \pm 0.04	1.64 \pm 0.03	1.66 \pm 0.02	1.74 \pm 0.04
	%BiNC	54.22 \pm 2.65	52.66 \pm 2.03	49.43 \pm 3.03	48.56 \pm 2.34
	BiMN	3.17 \pm 1.67	6.17 \pm 2.34	7.50 \pm 1.61	28.89 \pm 3.54 ⁺
	Mono MN	0.78 \pm 1.03	1.44 \pm 1.57	3.22 \pm 1.40	3.78 \pm 1.93
	NPBs	0.47 \pm 0.33	5.83 \pm 2.67	3.33 \pm 1.80	0.94 \pm 0.67
	Nuclear buds	1.00 \pm 0.82	2.50 \pm 2.06	2.00 \pm 1.63	0.75 \pm 0.33
	Patients < 50				
	NDI	1.39 \pm 0.45	1.45 \pm 0.03	1.52 \pm 0.04	1.31 \pm 0.02
	%BiNC	52.24 \pm 2.05	56.45 \pm 2.04	51.66 \pm 1.80	47.33 \pm 1.46
	BiMN	3.60 \pm 1.36	7.00 \pm 1.67	8.80 \pm 1.33	29.33 \pm 1.55 ⁺
	Mono MN	2.31 \pm 2.36	2.69 \pm 2.11	3.765 \pm 2.86	0.88 \pm 0.93
	NPBs	1.26 \pm 1	4.80 \pm 2.86	4.40 \pm 3.01	2.80 \pm 1.72
	Nuclear buds	1.20 \pm 0.6	0.20 \pm 0.40	1.40 \pm 1.102	1.80 \pm 1.17
	Patients 50-65				
	NDI	1.47 \pm 0.03	1.52 \pm 0.02	1.67 \pm 0.02	1.42 \pm 0.02
	%BiNC	54.45 \pm 2.45	52.26 \pm 1.34	53.26 \pm 3.44	42.42 \pm 2.21
	BiMN	7.77 \pm 3.09	8.23 \pm 3.12	13.46 \pm 3.1 ^{**}	31.92 \pm 4.98 ⁺
	Mono MN	1.75 \pm 1.64	3.50 \pm 2.50	1.50 \pm 1.80	4.38 \pm 2.52
	NPBs	1.54 \pm 1.39	5.69 \pm 2.58	5.77 \pm 2.91	2.77 \pm 2.04
	Nuclear buds	1.04 \pm 1.00	1.54 \pm 1.91	1.73 \pm 1.62	1.31 \pm 0.91
	Patients > 65				
	NDI	1.64 \pm 0.02	1.76 \pm 0.04	1.68 \pm 0.04	1.66 \pm 0.02
	%BiNC	56.65 \pm 2.06	53.65 \pm 3.54	52.66 \pm 2.64	42.26 \pm 2.12
	BiMN	9.69 \pm 2.52 [*]	9.87 \pm 3.33 [*]	13.86 \pm 2.50 ^{**}	31.29 \pm 5.16 ⁺
	Mono MN	3.67 \pm 2.43	2.33 \pm 1.25	4.33 \pm 1.60	7.33 \pm 1.80
	NPBs	1.28 \pm 1.07	5.93 \pm 2.28	5.07 \pm 2.49	1.43 \pm 1.40
	Nuclear buds	1.07 \pm 0.97	2.64 \pm 2.16	2.71 \pm 2.19	1.29 \pm 1.16

Footnote:

NDI: indicates nuclear division index

% Bi NC: indicates percentage binucleated cells in 1000 scored cells

BiMN: indicates number of micronuclei in 1000 binucleated cells

Mono MN: indicates micronuclei in mononucleated counted in 1000 cells.

NPBs: indicates number of nucleoplasmic bridges in 1000 cells

Table 5.5.4: Smoking habit as a confounding factor in the CBMN assay. Means \pm SD of different CBMN assay parameters of respiratory disease patients (lung cancer, COPD, asthma) and healthy controls in blood cultures treated with TiO₂ (5 and 10 μ g/ml) as well as a negative control of untreated blood cultures (Nc) and a positive control (Pc) of 0.4 μ M of mitomycin C (MMC). * $P < 0.05$, ** $P < 0.01$ and ⁺denotes highly significant when compared each group to its own untreated control as well as to corresponding healthy individuals untreated control.

Group type and confounder		TiO ₂ concentrations μ g/ml			Pc
		Nc	5	10	
Smoking	Non-smoker controls				
	NDI	1.74 \pm 0.02	1.62 \pm 0.02	1.68 \pm 0.02	1.62 \pm 0.05
	%BiNC	54.67 \pm 3.05	55.65 \pm 1.56	49.47 \pm 4.28	43.36 \pm 2.56
	BiMN	1.87 \pm 1.63	4.47 \pm 2.39	7.27 \pm 1.69	29.87 \pm 4.15 ⁺
	Mono MN	2.02 \pm 1.72	3.20 \pm 2.14	2.40 \pm 2.06	1.60 \pm 1.80
	NPBs	0.33 \pm 0.47	4.40 \pm 2.85	4.80 \pm 2.45	0.81 \pm 0.53
	Nuclear buds	0.67 \pm 0.79	2.13 \pm 1.59	2.13 \pm 1.50	0.72 \pm 0.47
	Non-smoker patient				
	NDI	1.77 \pm 0.04	1.64 \pm 0.03	1.66 \pm 0.06	1.47 \pm 0.03
	%BiNC	50.45 \pm 3.54	49.23 \pm 2.34	56.34 \pm 4.87	51.65 \pm 3.42
	BiMN	5.50 \pm 2.99	3.32 \pm 3.38	10.67 \pm 2.81 [*]	31.39 \pm 5.14 ⁺
	Mono MN	1.54 \pm 1.82	3.62 \pm 2.31	4.00 \pm 2.83	0.92 \pm 1.59
	NPBs	1.33 \pm 1.01	2.76 \pm 2.70	5.08 \pm 3.28	2.00 \pm 1.67
	Nuclear buds	0.75 \pm 1.16	1.83 \pm 1.77	2.00 \pm 1.87	1.58 \pm 1.11
	Smoker patient				
	NDI	1.31 \pm 0.03	1.52 \pm 2.56	1.67 \pm 0.02	1.61 \pm 0.03
	%BiNC	56.44 \pm 2.65	59.56 \pm 6.34	41.28 \pm 4.04	38.45 \pm 2.26
	BiMN	8.77 \pm 3.06	9.93 \pm 3.37 [*]	13.82 \pm 3.17 ^{**}	31.39 \pm 4.58 ⁺
	Mono MN	2.43 \pm 2.24	2.86 \pm 2.39	4.43 \pm 2.73	1.21 \pm 1.21
	NPBs	1.23 \pm 1.38	6.18 \pm 2.41	5.45 \pm 2.92	2.23 \pm 1.93
	Nuclear buds	1.14 \pm 0.75	1.86 \pm 2.12	2.09 \pm 1.95	1.32 \pm 1.02

Footnote:

NDI: indicates nuclear division index

% Bi NC: indicates percentage binucleated cells in 1000 scored cells

BiMN: indicates number of micronuclei in 1000 binucleated cells

Mono MN: indicates micronuclei in mononucleated counted in 1000 cells.

NPBs: indicates number of nucleoplasmic bridges in 1000 cells

Table 5.5.5: Ethnicity as a confounding factor in the CBMN assay. Means \pm SD of different CBMN assay parameters of respiratory disease patients (lung cancer, COPD, asthma) and healthy controls in blood cultures treated with TiO₂ (5 and 10 μ g/ml) as well as a negative control of untreated blood cultures (Nc) and a positive control (Pc) of 0.4 μ M of mitomycin C (MMC). * $P < 0.05$, ** $P < 0.01$ and ⁺denotes highly significant when compared each group to its own untreated control as well as to corresponding healthy individuals untreated control.

Group type and confounder		Nc	TiO ₂ concentrations μ g/ml		Pc
			5	10	
Ethnicity	Caucasian controls				
	NDI	1.83 \pm 0.08	1.56 \pm 0.04	1.52 \pm 0.03	1.68 \pm 0.06
	%BiNC	58.30 \pm 3.05	46.50 \pm 1.60	54.20 \pm 3.45	48.60 \pm 0.02
	BiMN	3.92 \pm 1.46	8.62 \pm 2.85	9.84 \pm 3.11	30.38 \pm 2.46 ⁺
	Mono MN	1.50 \pm 0.48	4.63 \pm 3.16	3.88 \pm 2.20	0.38 \pm 0.70
	NPBs	0.38 \pm 0.70	2.13 \pm 1.90	2.00 \pm 1.87	0.63 \pm 0.70
	Nuclear buds	0.38 \pm 1.80	1.75 \pm 1.20	4.50 \pm 1.41	5.25 \pm 2.73
	Caucasian patients				
	NDI	1.76 \pm 0.10	1.66 \pm 0.03	1.56 \pm 0.02	1.64 \pm 0.06
	%BiNC	52.66 \pm 2.65	55.23 \pm 1.26	47.88 \pm 2.65	56.26 \pm 2.44
	BiMN	9.96 \pm 1.46	8.62 \pm 2.85	12.24 \pm 3.11 [*]	39.54 \pm 1.25 ⁺
	Mono MN	1.7 \pm 1.55	1.35 \pm 1.82	2.95 \pm 2.64	3 \pm 2.47
	NPBs	1.46 \pm 1.35	3.24 \pm 1.44	5.65 \pm 2.85	1.3 \pm 1.31
	Nuclear buds	1 \pm 1.14	1.55 \pm 1.35	1.82 \pm 1.35	2.6 \pm 1.74
	Asian controls				
	NDI	1.75 \pm 0.04	1.74 \pm 0.03	1.88 \pm 0.02	1.67 \pm 0.01
	%BiNC	52.60 \pm 2.35	56.02 \pm 2.42	54.35 \pm 3.34	46 \pm 2.84
	BiMN	1.67 \pm 1.25	4.33 \pm 2.13	7.67 \pm 1.70	29.67 \pm 3.45 ⁺
	Mono MN	2.83 \pm 2.54	4.00 \pm 2.58	6.17 \pm 2.34	0.50 \pm 0.76
	NPBs	0.17 \pm 0.37	2.00 \pm 1.15	2.33 \pm 0.94	0.33 \pm 0.75
	Nuclear buds	1.75 \pm 0.04	1.74 \pm 0.03	1.88 \pm 0.02	1.67 \pm 0.01
	Asian patients				
	NDI	1.65 \pm 0.06	1.66 \pm 0.03	1.65 \pm 0.03	1.55 \pm 5.2
	%BiNC	54.70 \pm 2.34	52.50 \pm 4.56	56.34 \pm 3.42	37.35 \pm 2.34
	BiMN	4.67 \pm 1.25	8.40 \pm 3.98 [*]	10.40 \pm 1.36 ^{**}	31.20 \pm 5.42 ⁺
Mono MN	0.80 \pm 0.98	4.60 \pm 2.87	6.40 \pm 1.20	2.00 \pm 1.10	
NPBs	1.00 \pm 1.26	2.20 \pm 2.14	1.60 \pm 1.85	1.20 \pm 1.17	
Nuclear buds	2.40 \pm 1.36	4.00 \pm 3.58	2.80 \pm 2.48	1.60 \pm 1.50	

Footnote:

NDI: indicates nuclear division index

% Bi NC: indicates percentage binucleated cells in 1000 scored cells

BiMN: indicates number of micronuclei in 1000 binucleated cells

Mono MN: indicates micronuclei in mononucleated counted in 1000 cells.

NPBs: indicates number of nucleoplasmic bridges in 1000 cells

5.3.3 Ras p21 expression

The results of ras p21 expression are shown in table 5.6 and figures 5.3 A-D. A total of 20 samples were tested for ras p21 expression after treatment with different TiO₂ concentrations for 30 minutes (10, 30 and 50µg/ml) as well as a negative control of untreated lymphocytes. All 6 controls and 5 asthma patients' samples did not express ras p21 regardless of the fact they were treated and therefore TiO₂ did not modulate ras p21 expression in these groups. However, 5 COPD and 6 lung cancer patients expressed ras p21. The intensity of ras p21 bands was quantified with ImageJ software after scanning the western blotting X ray film. It has shown that the OD intensity of the bands alters in COPD patients which in some instances could be an up regulation of p21. On the other hand, there is a clear ras p21 reduction in OD in cancer patients after treatment with different TiO₂ concentrations.

According to the blots of ras p21 expression as shown in figure 5.3 A-D and the OD of the bands in table 5.6:

- None of the tested healthy controls or asthma patient groups showed clear proto oncogenic ras p21 expression.
- Of the eight COPD samples (fig. 5.3C) four individuals (COPD2, COPD5, COPD6 and COPD8) produce clear positive response of proto-oncogenic ras p21 expression, 2 (COPD1 and COPD4) had faint un measurable bands and 1 had a negative response (COPD 3). In COPD7 the bands disappeared after TiO₂ treatment.
- For lung cancer, six expressed measurable ras p21 bands (75%), 2 did not express ras p21 (negative) and in one sample (CA2) the bands disappeared

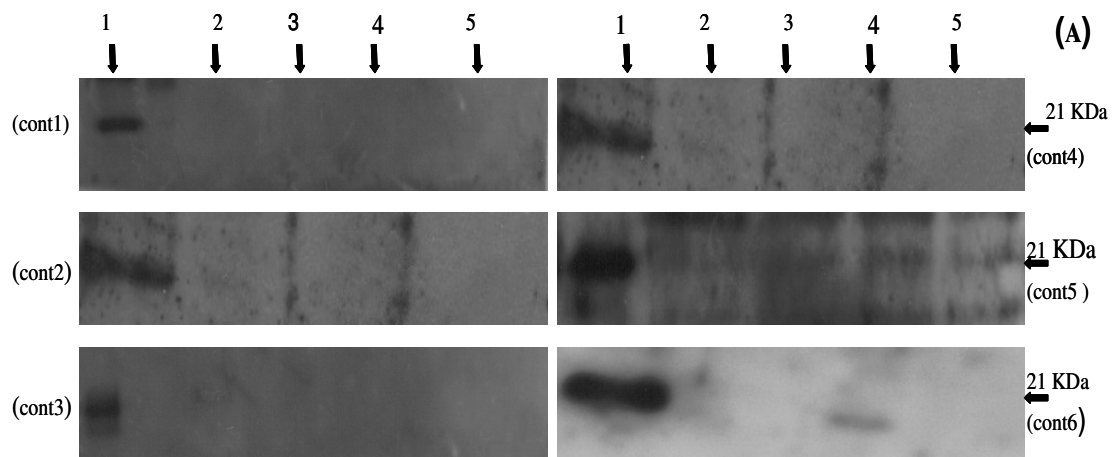


Figure 5.3A: Ras p21 protein expression in lymphocytes treated with different TiO_2 concentrations of (3)10 $\mu\text{g/ml}$, (4) 30 $\mu\text{g/ml}$ and (5) 50 $\mu\text{g/ml}$ for 30 minutes, in addition to (1) SW 480 cell lysate (positive control) and (2) untreated lymphocytes (negative control) in healthy controls (Cont),

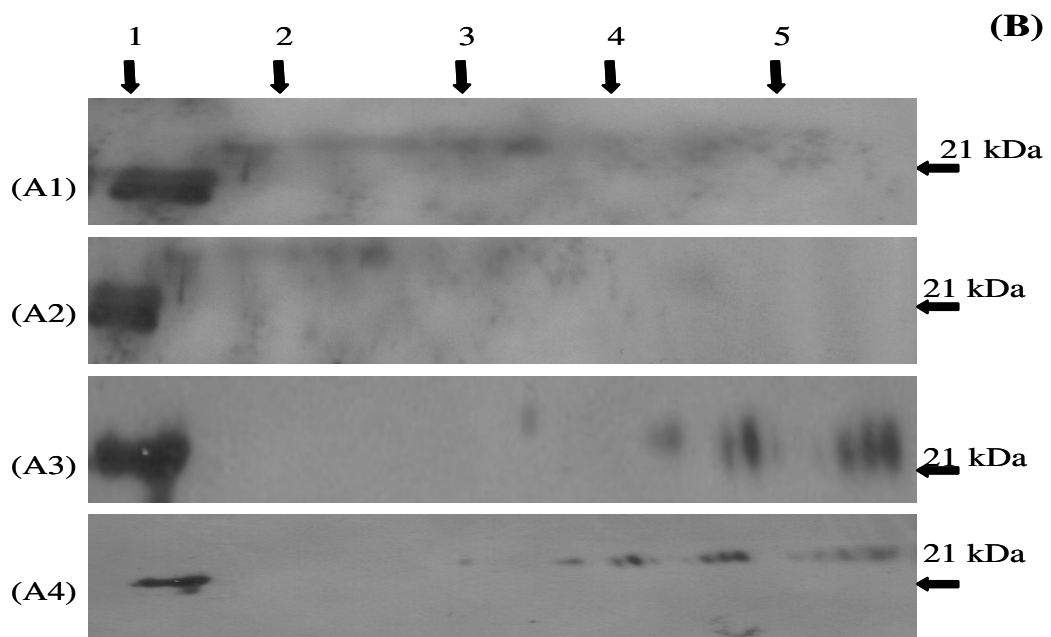


Figure 5.3B: Ras p21 protein expression in lymphocytes treated with different TiO_2 concentrations of (3)10 $\mu\text{g/ml}$, (4) 30 $\mu\text{g/ml}$ and (5) 50 $\mu\text{g/ml}$ for 30 minutes, in addition to (1) SW 480 cell lysate (positive control) and (2) untreated lymphocytes (negative control) in asthma (A) patients.

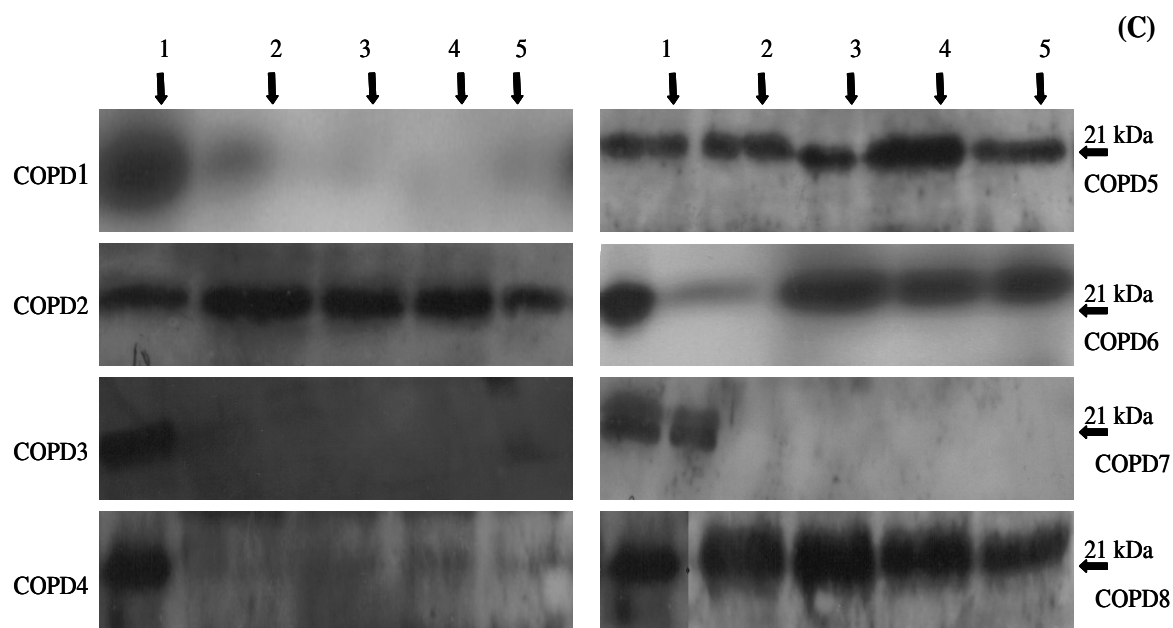
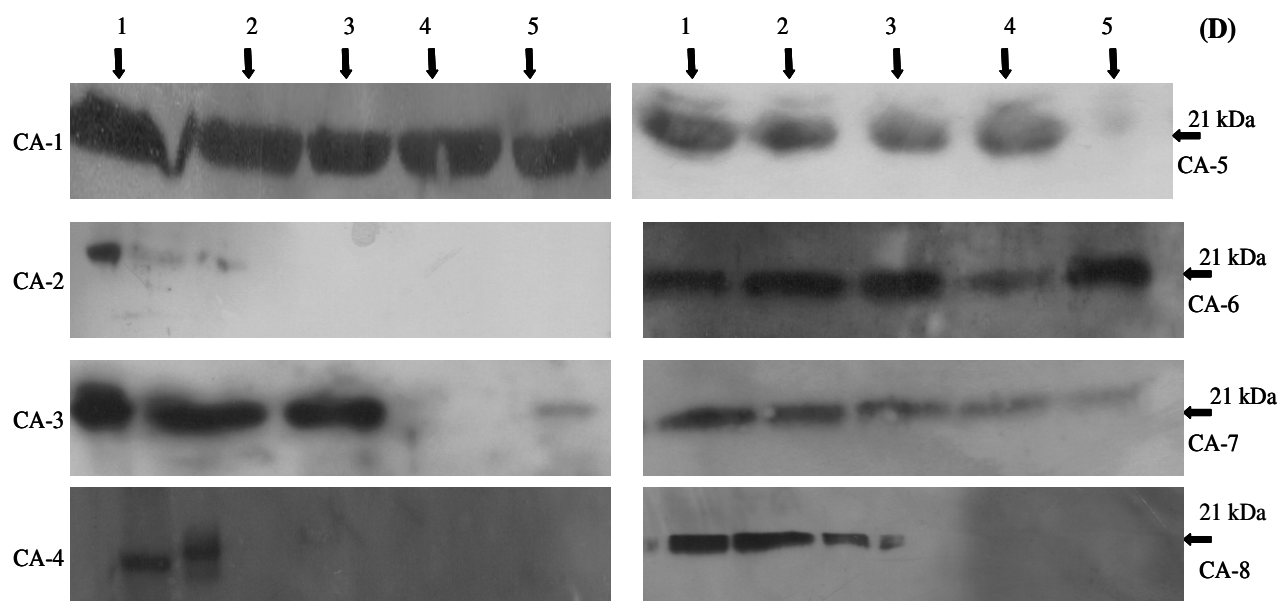


Figure 5.3C: Ras p21 protein expression in lymphocytes treated with different TiO_2 concentrations of (3)10 $\mu\text{g/ml}$, (4) 30 $\mu\text{g/ml}$ and (5) 50 $\mu\text{g/ml}$ for 30 minutes, in addition to (1) SW 480 cell lysate (positive control) and (2) untreated



lymphocytes (negative control) in COPD patients.

Figure 5.3D: Ras p21 protein expression in lymphocytes treated with different TiO_2 concentrations of (3)10 $\mu\text{g/ml}$, (4) 30 $\mu\text{g/ml}$ and (5) 50 $\mu\text{g/ml}$ for 30 minutes, in addition to (1) SW 480 cell lysate (positive control) and (2) untreated lymphocytes (negative control) in lung cancer patients (CA).

Table 5.6: Densitometry of ras p21 bands. The OD obtained with ImageJ software and standard deviations of scanned blots of ras p21 bands in respiratory disease patients and healthy controls lymphocyte protein extracts untreated (Nc) and treated with different TiO₂ concentrations (10, 30 and 50 µg/ml) for 30 minutes. All patients samples were coded * (OD means show ras p21 down regulation), \$ (OD means show ras p21 up regulation) and – (ras p21 not detected or immeasurable bands). Negative responses have not been included.

Group	Nc	TiO ₂ concentration in µg/ml		
		10	30	50
COPD 2	217.6 ± 23.9	212.6 ± 26.8	214.4 ± 15.5	187.3 ± 17.3
COPD 5	187.0 ± 23.3	220.7 ± 14.0	223.0 ± 18.0	221.8 ± 18.3
COPD 6 ^{\$}	55.1 ± 10.1	187.0 ± 32.5	184.5 ± 40.4	178.0 ± 18.3
COPD 7	227.0 ± 8.7	-	-	-
COPD	225.8 ± 5.8	222.0 ± 7.4	222.0 ± 7.4	219.6 ± 7.3
Lung cancer 1	222.0 ± 11.3	224.8 ± 15.6	226.6 ± 6.3	216.1 ± 9.4
Lung cancer 3*	227.1 ± 14.8	227.0 ± 22.7	-	-
Lung cancer 4	101.4 ± 25.4	-	-	-
Lung cancer 5	197.6 ± 9.3	196.7 ± 23.9	186.4 ± 32.6	-
Lung cancer 6	202.9 ± 18.0	201.5 ± 14.9	98.2 ± 32.9	197.2 ± 11.7
Lung cancer 7*	110.5 ± 20.3	75.2 ± 12.8	54.9 ± 11.6	43.9 ± 15.4
Lung cancer 8*	221.0 ± 22.7	217.7 ± 7.8	134.1 ± 13.9	-

5.4 Discussion

This *ex vivo/in-vitro* genotoxicity study of nanosized anatase TiO₂ was carried out on human lymphocyte cells from thirty six patients with different respiratory diseases namely lung cancer (sixteen), COPD (thirteen) and asthma (nine) plus fifteen healthy individuals as a control group. Investigations were carried out with the single-cell gel electrophoresis (Comet) assay and the cytokinesis-block micronucleus test.

Various *in vivo* and *in vitro* studies have reported cytotoxic and genotoxic effects in lung tissues (Duan et al., 2010; Hussain et al., 2010; Kim et al., 2010; Lamoureux et al., 2010; Larsen et al., 2010; Park et al., 2009; Schwaiger et al., 1991). None of the previous studies, however, have tested TiO₂ on respiratory disease patient lymphocytes. The Comet assay has been extensively used to measure DNA damage in isolated lymphocytes for oxidative DNA damage and repair capacity (Collins, 2009). For genotoxicity of TiO₂ NPs we used the Comet assay pH \geq 13 as a measurement of DNA strand breaks to quantify DNA damage (Collins and Horvathova, 2001; Olive and Banath, 2006). The Comet assay have been commonly used to examine the association of DNA damage and repair capacity in peripheral white blood cells with risk of various cancers (Schabath et al., 2003; Schmezer et al., 2001).

In this bio-monitoring study the effect of different TiO₂ concentrations (10, 30 and 50 µg/ml) on lymphocytes from respiratory disease patients and controls was examined in the alkaline Comet assay. The exposed lymphocytes have been shown to induce significant DNA strand breaks in respiratory disease patients compared to healthy individuals using Olive tail moment and % tail DNA. In addition, it was observed that the distribution of lymphocyte DNA damage was increased with age and smoking habit. However the mean increased only slightly and the possibility could be that only some of the cells exhibited an increase in DNA migration as Tice (Tice et al., 1991) observed that only 10-20% of the lymphocyte cells treated with bleomycin exhibited an increase in DNA migration.

In the present work, it has also observed that in the absence of photo-activation the lymphocytes of both controls and patients' lymphocytes exposed to different TiO₂ concentrations showed significant increases in DNA breakage, however, the patient groups showed more sensitivity to the lower concentration.

The results indicated that TiO₂ NPs at concentration levels between 10–50 µg/ml could cause dose dependent genotoxicity which was greater with the Olive tail moment than with % tail DNA. These findings were in line to some extent with previous studies that reported TiO₂ (anatase) in the absence of photo-activation, induced oxidative DNA damage to human bronchial epithelial cells (Gurr et al., 2005). Additionally previous studies with TiO₂ NPs have shown that, TiO₂ induced cytotoxicity and genotoxicity to human lymphoblastoid cells (Wang et al., 2007a), induced DNA damage to rat liver cells (Kocbek et al., 2010) and increased cell death in human neural (astrocytoma U87) cells (Hussain et al., 2006). Moreover, on *in vivo* study on mice with TiO₂ NPs in the

drinking water, has shown that the TiO₂ induced 8- hydroxy-2'-deoxyguanosine, induced γ -H2AX foci which is indicative of DNA double-strand breaks and additionally, induced micronuclei and DNA deletions (Trouiller et al., 2009). However, in a separate *in vitro* study with TiO₂ in human nasal epithelial cells, DNA strand breakage was not detected by the Comet assay (Hackenberg et al., 2010).

The photo-catalytic, reactive oxygen species (ROS) generation and *in vitro* cytotoxic properties of TiO₂ have previously been reported, following exposures to TiO₂ of human dermal fibroblasts and A549 human lung epithelial cells (Logan, 1983), besides, preceding studies reported genotoxic effects of TiO₂ on lymphocytes however, the mechanism of TiO₂ genotoxicity is still to be found.

Although the tendency of TiO₂ NPs to aggregate has been reported in this study (table 4.1) the fate of inhaled NPs is still unknown. This is mostly vital because of the fast growth in the promising nanomedicines and NPs are increasingly used in aerosols. There is possibility that NPs could become disaggregated after inhalation and subsequently deposited in the lung alveolar space. Thus they may enhance NPs inflammatory potency with subsequent recruitment of different inflammatory cells that could translocate to the interstitium which is anatomically the more vulnerable lung compartment and subsequently may lead to lung inflammation and fibrosis.

The unique properties of nanoparticles such as their small size and large surface area that increases their reactivity are the main incentives behind the rapid expansion of nanotechnology and which is specifically true for engineered NPs. The sizes, shapes and chemical compositions of NPs are diverse. While TiO₂ NPs have spherical shapes, carbon NPs has different shapes as nanotubes, nanowires and nanofibres. Sizes and

compositions additionally are different which could contribute to the variations in the toxicity of NPs.

Numerous *in vivo* studies have found that exposures to nanoscale particles produce greater genotoxic and cytotoxic effects compared to exposure to larger sized particles (Donaldson et al., 2002; Hackenberg et al., 2010), mostly because of the great potential of NPs to escape macrophages and therefore easily pass from the air space to the lung interstitium or to the blood vessels and thus either cause direct primary injury to the lungs such as interstitial inflammation and fibrosis, or translocate to other organs via the blood circulation and cause injury to other organs through oxidative stress production (Barillet et al., 2010; Narsimha Reddy et al., 2010). The NPs size effect was reported formerly in an *in vivo* study that compared the effect of different ultra-fine NPs (carbon black, TiO₂ and nickel) to their larger counterparts. The nano sized particles produced more compelling lung inflammation and cytotoxicity than larger sized particles in rats' lungs (Oberdorster et al., 2000; Oberdorster et al., 2005; Zhu et al., 2006). In addition, in a different *in vivo* instillation study with carbon black and TiO₂ it has been shown that the nano forms produced more inflammation than an equal mass of respirable larger size particles of the same material. Besides the NPs size, the surface area as well was found to have a key role to play in the process of the resulting inflammation that occurred (Roller and Pott, 2006; Wittmaack, 2007). Additionally, the leading size effect of engineered NPs on the lungs have been frequently reported and found to overload the lungs at lower burdens than the larger particles (Apley et al., 1971; Gilmour et al., 2004).

Transition metals in general have been shown to generate damaging hydroxyl radicals inside the cell which in response forms mutagenic adducts with DNA (Schins 2002;

Hillegass *et al.*, 2010). The oxidative stress generated inside the cell may cause lipid peroxidation in the cell which subsequently mediates such adduct formation (Vecchiet *et al.*, 1976). In general, NPs seem to be capable of crossing biological membranes and entering the cell nucleus instead of the cytoplasm to a greater extent compared to larger particles of the same material, and afterwards NPs could gain access to the nucleus and becomes closer to the DNA and consequently could cause genotoxicity from the NPs' oxidative products (Chen and von Mikecz, 2005; Geiser *et al.*, 2005).

A number of manufactured NPs have been found to encompass carcinogenic potentials. The potential for example of PM, asbestos and silica to cause cancer is well documented (Demircigil *et al.*, 2010; Heintz *et al.*, 2010; Olsson *et al.*, 2010). Two mechanisms have been postulated for NPs' carcinogenicity: the first is the direct genotoxic effect of the particles which involves the entrance of the particle to inside the cell with subsequent chemical and structural changes to the cell composition and therefore DNA damage, the second mechanism is by indirect genotoxic effects which are mediated through the particles' ability to induce inflammation. However, the exact mechanism is yet to be confirmed and might involve both mechanisms as well as TiO₂ which has the potential to mediate such effects (Kocbek *et al.*, 2010).

The inflammatory mechanism of NPs' carcinogenicity was previously proved on assays with carbon black which induced genotoxicity and lung tumors in rats. Such genotoxic effects and mutation on alveolar epithelial cells had occurred secondarily to substantial lung tissue inflammation that supported the inflammatory hypothesis of NPs carcinogenic outcome (Driscoll *et al.*, 1996).

As mentioned previously, with regard to the reported carcinogenicity of TiO₂ and other nanoparticles on the lungs, our objectives of carrying out this study was to assess and compare the effects of nanoparticles in peripheral blood lymphocytes from patients with respiratory diseases and healthy individuals using genotoxic endpoints.

In the Comet assay results have shown that in absence of UV irradiation, when each of the respiratory disease patient's and healthy controls lymphocyte cells were treated with different TiO₂ concentrations (10, 30 and 50 µg/ml) and compared to its own group untreated control, cells showed significantly different OTM values for all subjects. On the other hand, values were only genotoxic in the treated lymphocytes of all patient groups (lung cancer, COPD and asthma) and smokers group for both OTM and % tail DNA at all concentrations. The 30 µg/ml TiO₂ caused high DNA damage to all patient and control groups with exception of patients aged < 50. In this study the percentage tail DNA has shown less statistically significant values than the Olive tail moment. Both lung cancer and COPD showed less damage with the % tail DNA parameter than with OTM, and that the asthma subgroup showed the same pattern of significance as the healthy controls. For 50 µg/ml, TiO₂ was highly genotoxic to all patient and control groups, lung cancer, COPD, all patients and all control age groups and less genotoxic to male and female control groups when analysed separately ($P < 0.01$). In addition, significant differences between the patient and control groups when their confounding factors were compared (gender, age, smoking history and ethnicity) have been reported in the Comet assay, where the patient groups tend to have greater responses than the controls with the exception of patients aged < 50 which could be due to the fact all of the younger patients had asthma.

The rationale behind the use of CBMN assay to monitor the genotoxic effects of TiO₂ on different respiratory disease patients and control because CBMN assay is a genotoxicity assay which provides simultaneously substantial information on different chromosomal damage endpoints such as chromosomal breakage, rearrangements and gene amplification (Fenech, 2002b).

The measurement of micronucleus (MN) incidence in peripheral blood lymphocytes is broadly used in cytogenetics to estimate the presence and the scale of chromosomal damage in human populations exposed to genotoxic agents (Fenech et al., 1999). The reliability and low cost of the CBMN assay, has contributed to the global successful use and approval as a biomarker for *in vitro* and *in vivo* genome stability studies (Fenech, 2001). Chromosome loss, breakage and rearrangements and gene amplification play an important role in initiating events of the development and progression of cancer (Fenech, 2000; Reshmi et al., 2004), but less is known regarding the effect of TiO₂ exposure on the genome stability. We therefore focused on the effect of TiO₂ to help to find the effect of TiO₂ on the chromosomal stability of human lymphocytes. This could have important implication in the nanomedicines field in determination of the optimal concentration of TiO₂ that could be used without affecting genome stability.

In the current study, the frequencies of all CBMN assay parameters such as micronuclei, nucleoplasmic bridges and nuclear buds were higher in the lung cancer and COPD patients and the respiratory disease group as whole than in controls without being statistically significant.

The high basal DNA damage in lung cancer and COPD patients revealed in this study even before TiO₂ treatment have been reported previously as well. For example Cheng was reported higher spontaneous micronuclei levels in 42 lung cancer patients

compared to 55 controls (Cheng et al., 1996), beside that an investigation of genetic instability in lymphocyte cells of lung cancer patients with the Comet assay and micronucleus (MN) assay on 36 lung cancer patients and 30 controls have been previously carried out and the result in the MN assay indicated that the average values of MN in lung cancer patients were significantly higher than those of controls (El-Zein et al., 2006; Lou et al., 2007). In addition, Olive tail moment in the Comet assay was significantly higher than the controls, however; the difference in the tail length between the two groups was not significant (Lou et al., 2007). Furthermore, the basal DNA damage in COPD patients reported in a study measuring the genetic damage levels in COPD patients with CBMN and the Comet assays, revealed an increase in the basal DNA damage in COPD patients. As the frequency of nucleoplasmic bridges and buds was increased as well, the investigators contributed the increased frequency of micronuclei in COPD patients to clastogenic events and DNA amplification (Maluf et al., 2007).

In this study age, gender, smoking and ethnicity have been considered as confounding factors as recommended in previous bio-monitoring studies (Moller et al., 2000). An age related increase in responses by comparison with corresponding treated and untreated controls have also been reported in this study.

Male and female patients, as well had higher values than male and female controls which were increased in a concentration related manner. This result was in line with previous studies, as for example, inter-individual differences in DNA damage assessment carried out for the evaluation of DNA damage in urinary bladder washing cells in the Comet assay. The DNA migration was found to increase significantly with age in non-smokers (Gontijo et al., 2001). In another study on healthy Indian male and

female population, the results demonstrated a statistically significant higher level of basal DNA damage in males when compared to females (Bajpayee et al., 2002). In addition another large group study that involved 127 donors MN were increased with age and gender however, gender variation was mostly in people aged < 50 and women having higher MN frequencies (Wojda et al., 2007). With regard to ethnicity, Asians had generally less damage with Comet assay than Caucasians, but with MN assay Asian patients had a higher level of damage. These differences may be explained by difference in distribution of Asians in the groups. In the COPD groups there were none. In the lung cancer groups there was one Asian patient and in the asthma groups there were 6 out of 9, and in the control groups 5 out of 15. Furthermore, before treatment lung cancer and COPD patients showed basal values than asthma patients.

Although, cigarette smoking is a recognized cause of lung cancer, and passive smoking is an undisputed risk factor for developing lung cancer (24% increase in risk) 10% of lung cancers occurred in non-smokers (Brennan et al., 2000). Oxidative stress in active smokers correlates to the number of cigarettes smoked per day with decreased DNA repair capacity in smokers compared to non and ex-smokers (Raczka, 1981).

Regarding smoking habit as a confounding factor, the present work has revealed that smokers showed more damage than non-smoker patients in the Comet assay that in turn had higher values than non smoking controls. Furthermore smoking and non-smoking patients showed statistically significant increases in MN numbers when compared to the untreated controls of the healthy individuals, which was in line with a previous study that has shown smokers high basal DNA damage and low GSH cellular content which are the main intracellular defences against cigarette smoking (Fracasso et al., 2006), and in line with another study that was reported significant lymphocyte DNA damage in

cigarette factory workers measured in the Comet assay in smoking workers who smoked one or more cigarette per day as compared to non-smokers and Lam et al. who found that Smoking, male gender, age and even passive smoking increased DNA strand breaks in lymphocytes of elevator factory workers (Lam et al., 2002) . A similar finding was also previously reported (Fracasso et al., 2006).

However, these findings regarding smoking habit were in contrast to some other studies which did not find differences in genetic damage between subjects who smoked and non-smokers in the Comet parameters (Betti et al., 1995; Fracasso et al., 2002; Maluf et al., 2001; Touil et al., 2002; Undeger et al., 1999; Garaj-Vrhovac and Kopjar, 2003).

The ras proteins are guanine nucleotide-binding proteins. They play vital roles in the growth control of normal and transformed cells. Various growth factors and cytokines stimulate ras activation, which subsequently stimulates several downstream effectors, such as Raf-1/mitogen-activated protein kinase pathway. Mutated *ras* genes have been found in around 30% of human cancers, including lung, pancreatic and colon adenocarcinomas. The mutated *ras* gene produces mutated proteins that remain in an active state sending uncontrolled proliferative signals (Hezel et al., 2010). Oncogenes play key part in the development of many cancers because they regulate cellular proliferation. Cells have been transformed in culture by *ras* genes (Dimri et al., 2005; Liss et al., 2010; Takahashi et al., 2009). The *ras* genes encode the ras protein which consists of 189 amino acids of molecular weight of 21 kDa and are restricted to the inner surface of the plasma membrane. Many studies have targeted *ras* in the last two decades in order to have a better understanding of the mechanism by which mutated *ras* genes confer neoplasia in cells which could help in the production of more effective and

less toxic cancer therapeutics, and specifically target the defects in the cell growth control.

Immunoblotting studies in primary colorectal tumours and colon cancer cell lines treated with 10 μ M quercetin (a flavonoid plant, used as a nutritional supplement), have revealed that ras p21 expression was significantly down regulated by quercetin. Such findings suggested a possible chemo-preventive role for quercetin in colorectal carcinogenesis (Ranelletti et al., 2000) and showed that ras expression could be modulated by chemicals. Therefore, in the current study, we tested the effect of different TiO₂ NPs concentrations in lymphocytes of some of the study patients and controls to determine the ability of TiO₂ to modulate ras p21 expression. This is especially because ras p21 represents an important class of proto oncogene that are known to be associated with the development of lung cancer, particularly adenocarcinomas. Ras p21 is considered as a biomarker of some cancers including lung cancer because it is expressed in these cancers. In the present study, we found even after treatment with TiO₂ for 30 minutes lung cancer and COPD patients clearly expressed ras p21, however there was no expression of ras p21 in the asthma or control groups. Thus confirms a study by Anderson et al on a Polish population as many of the cancers and some of the COPD patients expressed ras p21 without any treatment (Anderson, 1999; Anderson et al., 1998b).

Overall, this study confirms in the absence of UV irradiation, that anatase TiO₂ of different sizes and concentrations gave positive responses in the Comet and MN assays. Moreover, anatase TiO₂ can induce DNA damage in the Comet assay, and increase the number of MN and NPBs without being statistically significant except when compared

with the untreated control of the healthy individuals. In addition, different levels of TiO₂ concentrations could modulate ras p21 expression

It is known from various studies (Blasiak et al., 2004; Kontogianni et al., 2007; Stoyanova et al., 2010), that untreated healthy control individuals have lower basal damage than patient groups. Thus it is not surprising that respiratory disease patients also have high basal damage than the healthy controls in the present study. It is difficult to disentangle the effect of TiO₂ from the effect due to smoking or the primary respiratory disease or any other confounder. However, in general lymphocytes from healthy individuals without confounding effects of smoking and respiratory disease show an increase of DNA damage with TiO₂ in the absence of UV irradiation (see chapter 3) (Gopalan et al 2009). These confounding responses could be working additionally synergistically or antagonistically. Overall, responses are still increased in the presence of TiO₂ regardless of confounding effects.

Chapter 6

General discussion

Nanotechnology as defined by the Centre for Responsible Nanotechnology (USA) is “the engineering of functional systems at the molecular scale. It is referred to the future ability to construct items from the bottom up, using techniques and tools being developed today to make complete, high performance products” (<http://www.crnano.org/whatis.htm>).

The U.S. National Science Foundation perspective for nanotechnology is as follows:

“Imagine a medical device that travels through the human body to seek out and destroy small clusters of cancerous cells before they can spread or a box no larger than a sugar cube that contains the entire contents of the Library of Congress or materials much lighter than steel that possess ten times as much strength”. (<http://www.scribd.com/doc/18303519/Nanotechnology-for-Communications>)

The future outlook for nanotechnology is to be a general-purpose technology (as it was called), because it will have significant effect on all other technologies and industries. The outcome as a result of nanotechnology as expected is that everything will become

better more effective medicines, enhanced stronger buildings, and cleaner safer water supply.

Although, nanotechnology has numerous useful applications in a variety of fields, many of these nanoparticles are non biodegradable and their long term behaviour is not well understood. In spite of the many promising useful medical applications, such as, imaging (Reddy et al., 2006), sensing (Lee and El-Sayed, 2006), gene delivery and artificial implants, multi-functional nanoparticles for cancer therapy and for organ or cell specific drug delivery through nanoparticles (Dobson, 2006), toxicology studies are still lacking.

Many of these particles can enter human tissues through different ports for example via the lungs after inhalation (Oberdorster, 2001), through the digestive system (Jani et al., 1990) or through the skin (Jeong et al., 2010; Lademann et al., 1999). Additionally, NPs are capable of crossing the body physiological barriers as for example the blood–brain barrier (Borm and Kreyling, 2004; Oberdorster et al., 2004) and the blood-testis barriers (Chen et al., 2009).

Another undesirable effect of nanotechnology is nano-pollution, which is the waste, generated during manufacturing of nanomaterials or by the nano-devices it produces. Nano-pollution could be very hazardous due to the particles small sizes which enable it to float easily in the air and get access to mammalian cells, which may have not yet adapted to the novel nano-waste environment because most of these wastes are man-made and do not appear normally in nature (Reynold et al., 2006).

Any free radical involving oxygen is referred to as a reactive oxygen species (ROS). DNA damage is an important issue in carcinogenesis; it is known that any agent capable

of chemically modifying DNA like ROS could be carcinogenic. The hydroxyl radical (OH^\bullet) attacks DNA and generates modified purines and pyrimidines bases. In healthy individuals, the steady level of oxidative DNA damage in normal cells is around 1 per 10^6 . This could explain the age related high incidence of cancer due to adducts of endogenous damage by ROS (Loft and Poulsen, 1996), many of which are known to be mutagenic free radicals produced in cells by cellular metabolism and by exogenous agents. These species react with bio-molecules in cells, including DNA. The resulting DNA damage caused by ROS or the oxidative damage is implicated to aging, mutagenesis, and carcinogenesis.

Many chemicals have negative impact on the germ cells as they can affect the germ cells directly or indirectly through their action on the somatic cells. Such effects include gametogenesis, fertility and offspring (Tran et al., 2007).

Exposure to such toxic chemicals and environmental contamination are an important cause of decline in male fertility and semen quality for example, industrialized countries have shown a 12% decrease in semen quality over the past 50 years (Carlsen et al., 1992). For example, dichlorobromopropane (DBCP) a soluble chemical resulted in lower sperm count and spermatogonial destruction (Potashnik et al., 1978). In addition, some chemicals were reported previously to preferentially accumulate in sperm cells compared to somatic cells with subsequent decrease in the number of sperm cells (Aoyagi et al., 2002).

Although the general impression that only maternal factor could result in foetal malformations *In vivo* studies in rats have disapproved such conception and found that mutations in male germ-line as a result of mutagens exposure such as radiations,

cytotoxic drugs and smoking could as well subsequently lead to congenital malformations in the foetus and subsequently may increase the risk of cancers (Anderson, 2005; Brinkworth, 2000).

Therefore, as a result a of increasing use of nanoparticles, one area of concern could be effect of nanoparticles on the germ cells, as these could have serious implications on the health of future generations. DNA damage to somatic cells can give rise to cancer but damage to germ cells could give rise not only to cancer, but also cause congenital defects in future generations. Hence it was decided to investigate the effect of ZnO and TiO₂ (anatase form) in human sperm and lymphocytes *in vitro* using the Comet assay because they are widely used ingredients in dermatological preparations and sunscreens and many other products.

Three different illumination conditions were used to investigate the genotoxic potential of the UV irradiated ZnO and TiO₂ (mean particle size 40-70 nm) using the Comet assay in lymphocytes and sperm cells in non illumination conditions (the dark, D), with pre-irradiation (PI) irradiation of the cells followed by treatment with either ZnO or TiO₂ and under simultaneous (SI) irradiation conditions in which cells simultaneously exposed to NPs and UV irradiation). It has been found in the absence of overt toxicity both ZnO and TiO₂ are capable of inducing genotoxic effects on human sperm and lymphocytes and the effect of ZnO and TiO₂ was enhanced by UVA. However, TiO₂ tended not to produce definite photo-genotoxic effects in sperm, although it appears to have definite photo-genotoxic effects with lymphocytes.

In spite of the small UV dose used in this study (1.68 mj/ cm²) compared to (700 mJ/cm² and 750 mJ/cm²) used in another study, both results are in line together (Dufour

et al., 2006). The parameters, which may have affected genotoxicity and / or photo-genotoxicity of ZnO and TiO₂ in this study include: a) In the dark, the cellular DNA damage could be due to the intrinsic genotoxic properties of ZnO and/ or TiO₂. b) DNA damage in pre- and simultaneously irradiated cells could be due to intrinsic genotoxicity or as a result of the effect of the UV system *per se* on the cells. The probable cause of the induced DNA damage could be that, oxygen radicals generated from ZnO and TiO₂ and acted synergistically with UVA to exacerbate the responses, as Many toxicological studies over the past years have raised concerns regarding the safety of nanoparticles as they readily generate oxidative stress through reactive oxygen species (ROS) because a strong link between nanoparticles and oxidative stress has been documented (Stone et al., 2007; Xia et al., 2006). In addition, many investigators have shown that ROS play a major role in nanoparticle-induced DNA damage (Donaldson and Stone, 2003). However, detailed molecular mechanisms involved in the cellular responses to genotoxic effects are not yet fully understood. Oxygen radicals can cause single and double strand breaks. However, the exact mechanism is yet to be known.

The expanding industrial and medical use of nanomaterials, especially zinc oxide and titanium dioxide, has led to growing concern about their toxicity. Accordingly, the intrinsic genotoxic and cytotoxic potential of these nanoparticles were evaluated using a HEp-2 cell line because they are the mainstay of cancer research for determining the cytotoxic effects of different chemicals (Cole et al., 1994; Kusumoto et al., 1988; Lebreton et al., 2008).

Cytotoxicity of ZnO and TiO₂ was tested with the mitochondrial activity (MTT) and the neutral red uptake (NRU) assays. The mitochondria and lysosome activity tests (MTT assay and NRU) indicated cytotoxicity of ZnO to HEp-2 cells treated beyond 4 h

reaching cell survival of < 16% after 48 hours. The highest TiO₂ concentration of 100 µg/ml was found to be cytotoxic to HEP-2 cells in less than 2 hours. The genotoxic potential of ZnO and TiO₂ in HEP-2 cells was also determined employing the Comet assay. It has been found that ZnO Genotoxicity in HEP-2 cells was seen within the Comet assay showing a 3-fold increase in DNA damage. On the other hand, TiO₂ treated HEP-2 cells showed a 2-fold increase in DNA damage after treatment with different TiO₂ concentrations. The genotoxic potential of ZnO and TiO₂ in HEP-2 cells was also investigated in the CBMN assay, and an increase in the formation of micronuclei was found after treatment with different concentrations ZnO and TiO₂ in HEP-2 cells.

As the oxidative stress could be one of the possible modes suggested for ZnO and TiO₂ induced DNA damage, it is as well now considered a major cellular signalling regulator and it was suggested that genotoxicity mediated by ROS could be further linked to other pathways (Schins and Knaapen, 2007); therefore, we investigated the tyrosine phosphorylation pathway additionally to the induction of DNA damage. These results have showed that, ZnO increased tyrosine phosphorylation of five proteins at 20, 21-22, 25, 30, and ~80 kDa with less effect of TiO₂ which showed almost similar bands pattern only at the highest concentration. Accordingly, tyrosine phosphorylation is modified by both ZnO and TiO₂ and produced genotoxic damage at two different end points in the Comet and CBMN assays in the absence of cytotoxicity. Such findings could be highly significant as previous studies showed, that dysregulation of protein tyrosine phosphorylation is associated with cell proliferation and the early stages of neoplasia (Blume-Jensen and Hunter, 2001; Hahn and Weinberg, 2002). On top of that, the increase in tyrosine phosphorylation of 21-22 kDa could be highly significant as a

correlation was previously found between cell transformation and tyrosine phosphorylation of 22-kDa protein (Glenney, 1989).

Phosphotyrosine phosphatases (PTPs) are important regulators of cellular signal transduction pathways. They are sensitive targets of oxidative stress and may be inhibited in conditions, which induce intracellular oxidation. The effects of PTPs inactivation amplified by activation of key protein tyrosine kinases (PTKs), lead to initiation of phosphotyrosine-signalling cascades, which are not under normal receptor control. These independent signals result in the accumulation of protein phosphotyrosine (Parker et al., 1998).

Signal transduction requires the actions of many signalling molecules, small changes in the activity of these molecules increased through signalling pathways. Therefore, the impairment of a function of a few oxidation-sensitive phosphotyrosine phosphatases (PTPs) may result in a shift in the balance of phosphotyrosine along a certain pathway. Communication between the cell surface and the nucleus might be altered along the signalling pathway, leading to message loss as the signal passes on. In cases of oxidative stress, inhibition of PTPs synergizes oxidative induction of protein tyrosine kinases (PTK) activity, and signalling cascades normally under receptor control are initiated in an independent manner.

Protein tyrosine phosphatase (PTP) inhibitory effect of treated cells has previously been reported, for example, PTP was found to inhibit tyrosine expression of sodium vanadate treated cells. This resulted in a bypass of growth arrest that occurred as the result of genotoxic exposure which indicates a role for tyrosine phosphorylation in the regulation of cell survival (Bae et al., 2009). Protein tyrosine phosphatase inhibition has also been previously reported in primary mammary epithelial cells, which enhanced cell survival

by decreasing apoptosis (Furlong et al., 2005). Additionally, earlier *in-vitro* studies on human U937 monocytes have shown that nano-cobalt increased the transcription of matrix metalloproteinases (MMP-2 and MMP-9) and protein tyrosine kinase (PTK) signalling pathways were involved through oxidative stress (Wan et al., 2008). As a result, we presumed that either PTP inhibition (Bae et al., 2009) or increased transcription of matrix metalloproteinases (MMP-2 and MMP-9) and PTK signalling pathways (Wan et al., 2008) could explain our findings of increased tyrosine phosphorylation as the result of genotoxic insult of ZnO and the highest concentration of TiO₂.

Prior studies have shown that nanoparticles produced an effect similar to irradiation by inducing carcinogenesis pathways through ROS-induced DNA damage, activating p53 and proteins related to DNA repair (Mroz et al., 2008). The acute DNA damage could trigger cell cycle arrest and leads to prolonged repair time or cell death (Friedberg and Meira, 2004). Accumulation of mutations, which are caused by excessive or incomplete DNA repair, is a known cause of oncogenesis (Rusyn et al., 2004).

In view of that, the present investigation conclusively provides further evidence for the genotoxic effect of ZnO and TiO₂ nanoparticles without UV irradiation in both the Comet assay and the micronucleus assay with induction of chromosomal damage at high, but non-cytotoxic concentrations. It also provides evidence of significantly increased chromosomal mutations at the highest concentrations which showed no cytotoxic effects in the NRU and MTT assays.

Here, an *ex vivo/in-vitro* study on human peripheral blood lymphocyte cells from thirty six patients with different respiratory diseases, lung cancer (sixteen), COPD (thirteen) and asthma (nine) in addition to fifteen healthy individuals as a control group, was

carried out to investigate the effect of nanosized anatase TiO₂ potential genotoxic effects with the single-cell gel electrophoresis (Comet) assay and the cytokinesis-block micronucleus test.

Although inhalation is unlikely for engineered nanomaterials compared with dust particles, this can take place during massive production and handling of NPs. Due to the chief effective deposition in the alveolar area, the crucial interactions of nanoparticles occur within the lung epithelial and alveolar macrophages. However, insufficient data currently exists on the mechanism of NPs permeability across the lung epithelium. A considerable number of studies showed that some of the NPs have the ability to induce lung inflammatory reactions; granulomas or even tumours e.g. TiO₂ in experimental animals.

Various *in vivo* studies in rats, have reported genotoxic and cytotoxic effects induced by different NPs in the lung tissues. As for example, aggregated ultra-fine TiO₂ have been shown to induce lung tumours in rats (Oberdorster, 1996; Bermudez et al., 2002), severe lung inflammations (Donaldson et al., 2002; Oberdorster et al., 2000), lung tumours (Heinrich et al., 1995) and progressive fibro-proliferative lesions and alveolar epithelial metaplasia (Bermudez et al., 2002). As for NPs in general, *in vivo* and *in vitro* studies have reported cytotoxic and genotoxic effects of different NPs in lung tissues (Duan et al., 2010; Hussain et al., 2010; Kim et al., 2010; Lamoureux et al., 2010; Larsen et al., 2010; Park et al., 2009; Schwaiger et al., 1991). None of the previous studies, however, have tested TiO₂ in the lymphocytes of respiratory disease patients.

In this study the effect of different TiO₂ concentrations (10-50 µg/ml) in lymphocytes from respiratory disease patients and controls was examined in the alkaline Comet

assay, then lymphocytes cells from each of the respiratory disease patients were treated with different TiO₂ concentrations and compared to its untreated controls using the Olive tail moment and % tail DNA.

In general, the exposed lymphocytes have been shown to induce significant DNA strand breaks in respiratory disease patients, and treated cells showed significantly different OTM values for all subjects. However for both OTM and % tail DNA, values were only genotoxic in the treated lymphocytes of all patient groups (lung cancer, COPD and asthma) and smokers group at all concentrations when compared to untreated controls.

Regarding the effect of different TiO₂ concentrations, the 30 µg/ml TiO₂ caused high DNA damage to all patient and control groups with the exception of those aged < 50. At 50 µg/ml, TiO₂ NPs were highly genotoxic to all patient and control groups, lung cancer, COPD, all patients and all control age groups and less genotoxic to male and female control groups when analysed separately.

In this work age, gender, smoking and ethnicity have been used as confounding factors as recommended in previous bio-monitoring studies (Moller et al., 2000). Accordingly, in the Comet assay, significant differences between the patient and control groups have been found when their confounding factors were compared to each other (gender, age and smoking history). It was observed that the distribution of lymphocyte DNA damage was increased with age and smoking habit in patient groups that tend to have greater responses than the controls with the exception of patients aged < 50 which could be due to the fact all of the younger patients had asthma who showed less or no basal DNA damage. Therefore, it could be reported that in the absence of photo-activation lymphocytes of both controls and patients' exposed to different TiO₂ concentrations

showed significant increases in DNA breakage. However, the patient groups showed more sensitivity to the lower concentration.

Furthermore, In the Comet assay it has been observed that the percentage tail DNA reported less statistically significant values than the Olive tail moment. Both lung cancer and COPD showed less damage with the % tail DNA parameter than with OTM, and that the asthma subgroup showed the same pattern of significance as the healthy controls.

Such findings were in line with previous studies in which TiO_2 in the absence of photoactivation induced oxidative DNA damage to human bronchial epithelial cells (ref). Moreover, TiO_2 NPs in drinking water induced DNA double-strand breaks, micronuclei and DNA deletions (Trouiller et al., 2009). Although, the photo-catalytic, reactive oxygen species generation and *in vitro* cytotoxic properties of TiO_2 have previously been reported, following human dermal fibroblasts and A549 human lung epithelial cells to TiO_2 (Logan, 1983). However, the exact mechanism of TiO_2 genotoxicity is still not fully determined.

The primary size effect of engineered NPs on the lungs have been frequently reported and found to overload the lungs at lower burdens than the larger particles (Apley et al., 1971; Gilmour et al., 2004). Such size inflammatory effect of NPs on the lung was also well observed in many *in vivo* studies in rats (Donaldson et al., 2002; Hackenberg et al., 2010; Oberdorster, 2001; Oberdorster et al., 2000; Zhu et al., 2006). This was mostly because of the great potential of NPs to escape macrophages and therefore easily pass from the air space to the lung interstitium or to the blood vessels and thus either cause direct primary injury to the lungs such as interstitial inflammation and fibrosis, or translocate to other organs via the blood circulation and cause injury through oxidative

stress production. Additionally, the surface area as well was found to have a key role to play in the process of the resulting inflammation that occurred (Roller and Pott, 2006; Wittmaack, 2007).

Although the tendency of TiO₂ NPs to aggregate have been noticed in this study (table 4.1), we do not have the knowledge regarding the fate of inhaled NPs in general which is vital because of the fast growth in nanomedicines and NPs are increasingly used in aerosols. Thus, NPs could become disaggregated and deposited in the lung alveolar space following inhalation that could enhance their inflammatory potency with subsequent recruitment of different inflammatory cells and subsequently may lead to lung inflammation and fibrosis.

In general, NPs could be capable of crossing biological membranes and entering the cell nucleus instead of the cytoplasm compared to larger particles of the same material, therefore, NPs could gain access to the nucleus and becomes closer to the DNA causing genotoxicity from their oxidative products (Chen and von Mikecz, 2005; Geiser et al., 2005).

A number of NPs have been found to have carcinogenic potential in the lungs, for example PM, asbestos and silica (Demircigil et al., 2010; Heintz et al., 2010; Olsson et al., 2010). As a result, one of the objectives to carry out this study was to assess and compare the effects of nanoparticles in peripheral blood lymphocytes from patients with respiratory diseases and healthy individuals using genotoxic endpoints.

The CBMN assay Was used to monitor the genotoxic effects of TiO₂ in different respiratory disease patients and control to estimate the presence and the scale of

chromosomal damage endpoints such as chromosomal breakage, rearrangements and gene amplification (Fenech, 2002b).

Chromosome loss, breakage and rearrangements and gene amplification play an important role in initiating events of the development and progression of cancer (Fenech, 2000; Reshmi et al., 2004), but less is known regarding the effect of TiO₂ exposure on the genome stability. It was therefore focused on the effect of TiO₂ on the chromosomal stability of human lymphocytes. This could have important implication in the nanomedicines field in determination of the optimal concentration of TiO₂ that could be used without affecting genome stability.

In general, in the CBMN assay, the lung cancer and COPD patients showed basal increases in MN values and the patients group had higher values compared to controls without being statistically significant. As for the confounding factors, an age related increase in responses by comparison with corresponding treated and untreated controls was reported in this study, which was in line with a previous study, that assessed an inter-individual differences in DNA damage by the Comet assay in which DNA migration was found to increase significantly with age (Gontijo et al., 2001). As for gender, male and female patients, as well had higher values than male and female controls, which were increased in a concentration related manner without being statistically significant.

Although, 10% of lung cancers occurred in non-smokers (Brennan et al., 2000), lung cancer is associated with cigarette smoking as well as passive smoking with a 24% increase in developing lung cancer and active smokers had decreased DNA repair capacity compared to non and ex smokers (Raczka, 1981). As a result, smoking habit have been used as a confounding factor in this study, where it was revealed that

smokers showed more damage than non smoker patients in the Comet assay and they in turn had higher values than non smoking controls. Additionally, smoking and non smoking patients showed statistically significant increases in MN numbers when compared to the control of the healthy individuals, which was in line with a previous study that has shown smokers high basal DNA damage and low GSH cellular content which are the main intracellular defences against cigarette smoking (Fracasso et al., 2006) , and in contrast to another studies that revealed smoking and gender were not significant confounding factors (Betti et al., 1995; Fracasso et al., 2002; Maluf et al., 2001; Touil et al., 2002; Undeger et al., 1999).

In the CBMN assay, we observed that lung cancer and COPD patients had high basal DNA damage before exposure to TiO₂ treatment. Such high basal DNA damage have been reported previously as well in a similar study where higher spontaneous micronuclei levels were reported in lung cancer patients compared to controls (Cheng et al., 1996), beside that on previous investigation of genetic stability in lymphocyte cells of lung cancer patients indicated that the average values of MN in lung cancer patients were significantly higher than those of controls (El-Zein et al., 2006; Lou et al., 2007). As for COPD patients prior studies reported an increase in the basal DNA damage in COPD patients with increasing values of all CBMN assay parameters (Maluf et al., 2007). In addition, different levels of TiO₂ concentrations could modulate ras p21 expression.

Future perspectives

Engineered nanoparticles (NPs) cover a variety of classes of technological materials with novel properties. Such unique properties make NPs more attractive for many uses in pharmacology and biomedicine.

There is strong evidence linking NPs toxicity to their unique properties such as their size, surface area and other novel chemical properties that attracted the nanotechnology. Additionally, most of the nanomaterials react according to oxidative stress assumption, but possible nanomaterials connection with biological systems may result in more forms of damage.

In the literature, there is a growing support that some nanomaterials are potentially harmful to humans; accordingly, firm industrial safety methods should be available to limit contact during nanomaterials manoeuvring. Moreover, given the doubt about the nanomaterials capability to induce significant pathogenetic effects, a toxicological evaluation of each nano-material should be carried out.

Nanotechnology holds great promises for medicine, manufacturing and for the environment including efficient water purification and removal of toxic waste. Currently, nanoparticles like ZnO and TiO₂ are already widely used in many consumer products. The variety of applications ranges from using nanoparticles within sunscreens, pigments, tooth pastes, antiseptic coatings, paints and the coating of spectacles to make products unbreakable and scratchproof. As a result, the evaluation of the toxic potential of nanoparticles within their substrate is imperative. However, in many cases detailed toxicological evaluation of nanoparticles including their genotoxic assessment is lagging behind the technological application of medical and consumer nano-products. Thus, in the very near future the focus will shift from nanotechnology towards nanotoxicology. Recent studies and our study have shown that under certain conditions nanoparticles can increase genotoxic damage within cells and potentially lead to adverse effects. Nanotechnology and Nanomedicines are promising more medical benefits within the next years. This includes the discovery of nano-based drugs and nanoscale

diagnostic devices. The Cancer research has a goal of developing nanometer scale multifunctional nano therapeutic products which can diagnose, treat and monitor cancer progress. These products include contrast agents to improve cancer cells resolution to be detected at a single cell level. However, toxicity of nanoparticles should not be ignored. Therefore it is essential that deep research should be carried out for nanomedicines achievements to become real success. Human's exposure to airborne nanosized particles has increased considerably over the last century due to the rapidly expanding field of nanotechnology. Therefore, it is probable to become another source of exposure through different routes primarily through inhalation then other different routes such as skin uptake, ingestion or injection of engineered nanomaterials.

In the meantime, the current information regarding engineered nanomaterials prospective hazards still lacking. Toxicology studies results of NPs and airborne ultra-fine particles should be taken seriously as a base for nanotechnology expansion. Collectively, some emerging concepts of nanotoxicology can be identified from the results of these studies.

Appendices

Appendix 1A)



| Participant Information Sheet for patients (Version 3, [07-07-09](#))

Study title: Effect of small (nano) particles on white blood cells in patients with chest diseases compared to white blood cells in people without chest diseases.

**Reviewed by Leeds Central Research Ethics Committee (REC)
(REC reference number: 09/H1313/37)**

Invitation to the research study

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish to and you will be allowed around 24hours to consider this.

(Part 1 tells you the purpose of this study and what will happen to you if you take part. Part 2 gives you more detailed information about the conduct of the study).

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?

In this study white blood cells will be treated in a test tube with very small chemical particles to determine if patients with respiratory diseases are more at risk after exposure. A blood sample of around 6-8 teaspoons (40 ml) will be taken. Samples will be stored only for the duration of the study (about 3 years) and used for studies of a similar nature or to check original responses. The research is for a PhD programme.

Why have I been invited?

Because you are a patient at the Clinic for chest diseases and we should like to determine if these small chemical particles could be more harmful to you than to people without chest diseases.

Do I have to take part?

It is up to you to decide. We shall outline the study and go through this information sheet, which we shall then give to you. We shall ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

Part 2

What will happen to me if I take part?

A single blood sample will be taken and you will not need to attend the Clinic again for this research study. A brief questionnaire will need to be completed by the researchers.

We shall need to access your notes so that they can be linked in an anonymous way to your clinical data which can be tied up with the research results. Each individual will be given a coded study number.

The data obtained will only be available to the research team and will **not** be returned to you. Responses will be compared only on group basis i.e. collective responses from patients with chest diseases compared to collective responses from people without chest diseases. Results could be published in the form of scientific papers. The work will benefit the medical and scientific community at large, but will not be of direct benefit to you as an individual. If, however, you would like more information, Dr BK Jacobs will be prepared to talk to you individually about study results.

People who cannot take part in the study.

People who are not well enough to take part will be excluded (e.g. those with anaemia)

Appendix 1B

DATA COLLECTION FORM

(To be completed by the Doctor)

STUDY TITLE: EFFECT OF SMALL PARTICLES (NANO) ON WHITE BLOOD CELLS IN PATIENTS WITH CHEST DISEASES COMPARED TO PEOPLE WITHOUT CHEST DISEASES.

**REVIEWED BY LEEDS CENTRAL RESEARCH ETHICS COMMITTEE (REC)
(REC REFERENCE NUMBER: 09/H1313/37)**

PATIENT NUMBER DATE OF SAMPLE

AGE

SEX (PLEASE TICK) ☐ M ☐ F CONSENT ☐ Y / N
ETHNIC GROUP INFORMATION SHEET ☐ Y / N

OCCUPATION

CURRENT SMOKER ☐ Y/N PAST SMOKER ☐ Y/N HOW MANY/MUCH PER DAY?
CIGARETTES CIGARS PIPE
ALCOHOL ☐ Y/N UNITS PER WEEK

DIET ☐ WESTERN ☐ ASIAN ☐ OMNIVORE ☐ VEGETARIAN ☐ VEGAN

VITAMINS / ANTI-OXIDANTS
(PLEASE LIST)

PRESCRIBED DRUG USE
(PLEASE LIST)

RECREATIONAL DRUG USE ☐ Y/N

IF YES PLEASE LIST

MEDICAL

LUNG CANCER |
COPD | ASTHMA
EXTENT SITE HISTOLOGY SURGERY

LUNG CANCER
COPD
ASTHMA
OTHER MEDICAL CONDITIONS
(PLEASE LIST)
FAMILY HISTORY OF CANCER AND
LUNG CANCER, COPD, ASTHMA

Centre Number:

CONSENT FORM FOR PATIENTS

Title of Project: **Effect of small (nano) particles on white blood cells in patients with chest diseases compared to white blood cells in people without chest diseases. (Version 3, 07- 07-09)**

**Reviewed by Leeds Central Research Ethics Committee (REC)
(REC reference number: 09/H1313/37)**

box

1. I confirm that I have read and understand the information sheet (version 3, 19-06-09) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the NHS Trust or the University of Bradford, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.
4. I agree that the sample I have given and the information gathered about me can be stored at the University of Bradford, as described in the attached information sheet.
5. I agree to take part in the above study.

☐
☐
☐
☐
☐

Name of Patient

Date

Signature

Name of Person
taking

Date

Signature

consent

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Appendix 1 D)

Participant Information Sheet for healthy volunteers (Version 3, 19-06-09)

Study title: Effect of small (nano) particles on white blood cells in patients with chest diseases compared to white blood cells in people without chest diseases.

**Reviewed by Leeds Central Research Ethics Committee (REC)
(REC reference number: 09/H1313/37)**

Invitation to the research study

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish to and you will be allowed around 24 hours to consider this.

(Part 1 tells you the purpose of this study and what will happen to you if you take part.

Part 2 gives you more detailed information about the conduct of the study).

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?

In this study white blood cells will be treated in a test tube with very small chemical particles to determine if patients with respiratory diseases are more at risk after exposure. A blood sample of around 6-8 teaspoons (40 ml) will be taken. Samples will be stored only for the duration of the study (about 3 years) and used for studies of a similar nature or to check original responses. The research is for a PhD programme.

Why have I been invited?

You have been invited because you don't have a chest disease and we should like to determine if these small chemical particles could be more harmful to people with chest diseases than those without chest diseases.

Do I have to take part?

It is up to you to decide. We shall outline the study and go through this information sheet, which we shall then give to you. We shall ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason.

Part 2

What will happen to me if I take part?

Only a single blood sample will be taken for this research study. A brief questionnaire will need to be completed by the researchers.

Each individual will be given a coded study number so that your clinical data is linked in an anonymous way with the research results.

The data obtained will only be available to the research team and will **not** be returned to you. Responses will be compared only on group basis i.e. collective responses from patients with chest diseases compared to collective responses from people without chest diseases. Results

could be published in the form of scientific papers. The work will benefit the medical and scientific community at large, but will not be of direct benefit to you as an individual. If, however, you would like more information, Dr BK Jacobs will be prepared to talk to you individually about study results.

People who cannot take part in the study.

People who are not well enough to take part will be excluded (e.g. those with anaemia).

If you have any further questions, you could contact the research team:

Dr B K Jacobs, Consultant Respiratory Medicine, MB, CHB, MD, FRCP, Bradford Hospitals NHS Trust, Bradford Royal Infirmary, Duckworth Lane, Bradford, BD9 6RJ.
Telephone: 01274-366 832

Centre Number:

CONSENT FORM FOR HEALTHY VOLUNTEERS

Title of Project: **Effect of small (nano) particles on white blood cells in patients with chest diseases compared to white blood cells in people without chest diseases. (Version 3, 07-07-09)**

**Reviewed by Leeds Central Research Ethics Committee (REC)
(REC reference number: 09/H1313/37)**

initial

Please

box

6. I confirm that I have read and understand the information sheet (version 3, 19-06-09) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.
7. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.
8. I agree that the sample I have given and the information gathered about me can be stored at the University of Bradford, as described in the attached
9. Information sheet.
10. I agree to take part in the above study.

☐
☐
☐
☐

Name of Volunteer

Date

Signature

Name of Person
taking

Date

Signature

consent

When completed, 1 for patient; 1 for researcher site file;

Appendix 2)

Publications

1. Gopalan, R. C., Osman, I. F., et al. (2009). "The effect of zinc oxide and titanium dioxide nanoparticles in the Comet assay with UVA photoactivation of human sperm and lymphocytes." Nanotoxicology 3(1): 33-39.
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